

**THE EFFECT OF THE BOORoola FECUNDITY GENE
ON THE CONTROL OF OVARIAN FUNCTION IN SHEEP**

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ABSTRACT

Ewes possessing the single, or major, Booroola fecundity (Fec^B) gene have higher ovulation rates than non-carriers of the Fec^B gene. Previous investigations into the hormonal control of ovulation rate in ewes possessing the Booroola fecundity (Fec^B) gene have been complicated by comparisons of animals of different strains, or by using animals which were of an unknown genetic background. The establishment of breeding lines has enabled animals to be chosen whose pedigree is well documented and whose genetic relationship to other members within the same experimental group can be carefully regulated. The work in this thesis has investigated gene specific differences in FSH, inhibin, oestradiol and IGF-1 as possible agents in promoting prolificacy in two populations (F_2 and backcross) of Booroola Scottish Blackface ewes which differ only in the major Booroola gene and closely related genes.

In both populations, 80% of ewes were induced to ovulate during seasonal anoestrus in response to a single i.m. injection of hCG (750 i.u.) confirming that the mechanisms controlling ovulation rate are still present in the non-breeding season. During the breeding season and seasonal anoestrus, Fec^BFec^B ewes had higher ovulation rates and higher peripheral FSH concentrations compared to Fec^+Fec^+ ewes from the F_2 population. However, FSH concentrations did not differ between Fec^BFec^+ and Fec^+Fec^+ ewes from either F_2 or backcross populations, despite significant differences in breeding season ovulation rate. In ovariectomized ewes, FSH concentrations were similar between Fec^BFec^+ and Fec^+Fec^+ ewes from both populations, but significantly higher in Fec^BFec^B ewes, supporting the theory of genotypic differences in pituitary gland release.

Since genotypic differences in FSH concentration were not always associated with genotypic differences in ovulation rate, the hypothesis that there was a difference in follicular responsiveness to FSH, in terms of growth and/or oestradiol production, was tested by the use of an *in vitro* culture system. There were no genotypic differences in growth or oestradiol production by small follicles in response to FSH *in vitro* from either population, although the possibility that such differences exist towards the latter stages of follicular development cannot be excluded. A novel *in vivo* culture system using ovariectomized SCID mouse transplanted with ovine ovarian cortex containing primordial and small follicles was developed to determine if follicular growth could be maintained through to the latter stages of development. The xenografting technique produced antral, although not Graafian, follicles and may prove to be a useful model to investigate the mechanisms responsible for the initiation of follicular growth. However, the production of follicles within the xenograft was variable and so genotypic differences in follicular development could not be demonstrated.

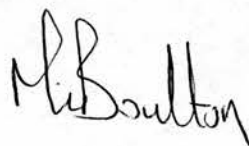
Studies designed to investigate other factors responsible for controlling the genotypic differences in ovulation rate showed that peripheral IGF-1 concentrations during the breeding season did not differ between the genotypes in the F₂ population. IGF-1 did increase during the follicular phase of the oestrous cycle suggesting acute regulation by the ovary, possibly through the action of oestradiol, although ovariectomy did not alter basal IGF-1 concentrations showing the ovary is not a major source of IGF-1. Investigation of ovarian secretion rates of inhibin revealed Fec^BFec^B ewes had significantly lower concentrations than either Fec^BFec⁺ or Fec⁺Fec⁺ ewes from the F₂ population. This finding supported

the hypothesis that peripheral FSH and inhibin are inversely related to each other. In view of genotypic differences in peripheral inhibin, it was surprising to find no genotypic differences in follicular fluid concentrations of either inhibin or ovarian secretion of oestradiol, which acts synergistically with inhibin to inhibit FSH secretion from the pituitary.

In conclusion, there were genotypic differences in both peripheral inhibin and FSH concentrations, however FSH was not wholly responsible for controlling genotypic differences in ovulation rate. Peripheral measurements of IGF-1 and oestradiol failed to show differences between the genotypes. The Booroola fecundity gene appears to be acting both at the level of the ovary and the pituitary gland, although the predominant site of action has yet to be determined.

DECLARATION

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the designing, preparation and execution of this thesis.

A handwritten signature in dark ink, appearing to read 'M. I. Boulton', written in a cursive style.

Maragret Iona Boulton

PUBLICATIONS ARISING FROM THE THESIS

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Boulton, M.I., Haley, C.S. and Webb, R. (1993). The effect of the Booroola Fecundity (Fec^B) gene on peripheral Follicle stimulating hormone (FSH) concentrations and ovulation rates during oestrus, seasonal anoestrus and on FSH concentrations following ovariectomy in Scottish Blackface ewes. Submitted to *J. Reprod. Fert.*

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CHAPTER 1

Literature review: The reproductive physiology of the ewe

1.1. The seasonally anoestrous ewe

The annual reproductive cycle of the ewe consists of a breeding and non-breeding season, the latter often termed 'seasonal anoestrus'. For breeds located in the northern hemisphere, the non-breeding season normally commences from late spring through to late summer and is characterized by the absence of regular ovarian cycles (Yeates, 1949; Hafez, 1952).

1.1.1. Temporal characteristics of the non-breeding season

Most of the essential components of the reproductive axis remain functional during seasonal anoestrus in the sheep. Ovarian follicles develop, and can ovulate (Wallace, McNeilly and Baird, 1986; Webb, 1988; Driancourt and Fry, 1990; Webb, Baxter, McBride, Richie and Springbett, 1992b), steroids are produced and gonadotrophic hormones secreted (Cole and Miller, 1935; Hutchinson and Robertson, 1966; Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969; Symons, Cunningham and Saba, 1973; Foster and Crighton, 1974; Karsch and Foster, 1975; Scaramuzzi and Baird, 1977; Pearce and Oldham, 1988). The viability of the induced corpora lutea during seasonal anoestrus has been reported to be lower (Crighton, Foster, Holland and Jeffcoate, 1973; McNeilly and Land, 1979), although this may be dependent on breed (Webb, personal communication). This reduced lifespan of the induced corpora lutea probably reflects either defects in hormonal priming of the preovulatory follicle and/or inappropriate luteotrophic support after ovulation has occurred resulting in premature

induction of luteolysis (Hunter, 1991; Wallace, Ashworth, Aitken and Cheyne, 1992). During seasonal anoestrus, peripheral progesterone levels are near detection limits in the absence of a corpus luteum, whilst follicle stimulating hormone (FSH) concentrations are similar to the breeding season (Walton, McNeilly, McNeilly and Cunningham, 1977; see McNeilly, 1980 for review). Prolactin concentrations during seasonal anoestrus, however, are elevated compared to the breeding season (Walton, Evins, Fitzgerald and Cunningham, 1980; see Curlewis, 1992 for review), but are seemingly uninvolved in the determination of the reproductive season (see McNeilly, 1980 for review).

In ewes, the seasonal anoestrous period is characterized by low frequency pulses of hypothalamic gonadotrophin-releasing hormone (GnRH) which are higher in amplitude than during the breeding season (Clarke, 1988; Barrell, Moenter, Caraty and Karsch, 1992). The GnRH pulses induce pulses of luteinizing hormone (LH) from the pituitary gland. In turn, the discrete pulsatile discharge of LH causes an increase in the rate of ovarian oestradiol secretion, similar to that of the oestrous cycle, suggesting that ovarian follicles are capable of responding to tonic LH concentrations (Scaramuzzi and Baird, 1977). The cause for breeding inactivity is thought to be primarily due to the lack of persistent elevated LH concentrations, which prevent a sustained rise in oestradiol concentrations and disruption of the LH surge for behavioural oestrus and ovulation to take place respectively (see Legan and Karsch, 1979 for review). Indeed, during seasonal anoestrus increases in LH levels are inhibited by the rise in oestradiol concentration that is a consequence of the increase in LH (a phenomena called 'negative feedback'). As a result of the oestradiol-induced inhibition of LH, the concentrations of oestradiol decrease to a minimum threshold, whereupon LH secretion is again

triggered and the process of negative feedback continues. The negative feedback relationship between LH and oestradiol in seasonal anoestrous sheep is in sharp contrast to the positive feedback relationship that exists between the two hormones during the oestrous cycle (Legan, Karsch and Foster, 1977; Karsch, Goodman and Legan, 1980; Goodman and Karsch, 1981; ^{I'Anson} Goodman, Bittman, Foster and Karsch, 1982; Legan, L'Anson, Fitzgerald and Fitzovich, 1985).

The ability of the ewe to mount a sustained increase in GnRH pulse frequency following the withdrawal of progesterone concentrations represents a fundamental difference between the breeding and anoestrous seasons (Karsch, Bittman, Foster, Goodman, Legan and Robinson, 1984; Legan, L'Anson, Fitzgerald and Akaydin, 1985). During seasonal anoestrus, despite the absence of cyclic gonadotrophin surges, the fundamental basis for seasonality does not rest in the surge-generating mechanism, because surges of GnRH and LH can be induced, given the appropriate oestradiol stimulus (Goodman, Legan, Ryan, Foster and Karsch, 1981; Clarke, 1988; Moenter, Caraty and Karsch, 1990). The neuroendocrine basis for seasonality therefore resides with the GnRH pulse generating system (Barrell *et al.*, 1992).

The type of feedback action of ovarian oestradiol on elevated LH concentrations is dependent on photoperiod (Hoffmann, 1973; Legan and Karsch, 1979), although the exact nature of this relationship may well be governed by an inherent physiological mechanism (Owen, 1976; Malpaux, Robinson, Wayne and Karsch, 1989). The sheep is defined as a 'short day breeder' and during seasonal anoestrus, when the daylength is long, photoreceptors detect light cues which are relayed through a monosynaptic nerve tract to the suprachiasmatic nuclei of the hypothalamus. The suprachiasmatic nuclei of the hypothalamus, after

receiving input from the circadian system, transmit this photoperiodic message by way of the superior cervical ganglia to the pineal gland. The pineal gland nocturnally produces an hormonal signal, in the form of melatonin, in response to this neural input, and the duration, or phase, of this hormones secretion serves as a code for daylength (Rollag and Niswender, 1976). The melatonin code can be interpreted as either inductive, resulting in breeding activity, or suppressive resulting in seasonal anoestrus (see Karsch *et al.*, 1980; see Karsch *et al.*, 1984 for review; Robinson, Radford and Karsch, 1985). Once the specific melatonin pattern is set, the photosexual response becomes independent of daylength that is 'photorefractoriness' occurs (Bittman, Karsch and Hopkins, 1983). Seasonal anoestrus is due to both photorefractoriness and photosuppression, although the relative importance of these two components varies with stage of anoestrus (Kao, Schaeffer and Jackson, 1992). The role of thyroid hormones in the control of seasonality in the ewe have yet to be fully elucidated, but are shown to be involved by thyroidectomy which prolongs the breeding season (see Wilmut, Haley, Simons and Webb, 1992 for review). The thyroid hormones may be involved in the formation of synapses on GnRH neurones which are known to be fewer in number during seasonal anoestrus (Moenter, Woodfill and Karsch, 1991).

The transition from seasonal anoestrus to the normal cyclicity associated with the breeding season is marked by ovulation and corpora lutea formation, which are unaccompanied by behavioural oestrus and are thus termed 'silent heats' (Grant, 1933; Cole and Miller, 1935). The progesterone produced from these first corpora lutea sensitize the hypothalamus to oestradiol so that behavioural oestrus can occur in future cycles (Robinson, 1959).

1.2. The oestrous cycle

The domestic ewe is seasonally polyoestrus and for any breed, the duration of the breeding season may be considerably modified according to geographical location, climatic environment and nutritional state. The length of the oestrous cycle is 16-17 days (McKenzie and Terrill, 1937; Hafez, 1952) and is more variable in the second half of the breeding season, as a result of the luteal phase being extended (Hammond, 1944). The length of the oestrous cycle is not significantly different between breeds which differ in prolificacy, for instance the naturally multi-ovulatory Booroola Merino is known to have an oestrous cycle length that is similar to Merinos, that is 16 ± 0.2 days (Bindon and Piper, 1982). Prolific breeds are those which exhibit high ovulation rates. For domestic species, the oestrous cycle is categorised into two parts, namely the luteal and follicular phases (see Robertson, 1977; Gordon, 1983 for review). For the majority of sheep breeds the follicular phase culminates in the ovulation of normally one-two follicles. In the sheep, similar to the monkey (Gougeon, Lefèvre and Testart, 1992), both ovaries have an equal capacity to ovulate (Wheeler, 1978), which is unlike the cow, where the right ovary predominates (Reece and Turner, 1937; see Arthur, Noakes, Pearson, 1982 for review).

1.2.1. Temporal characteristics of the luteal phase

In the ewe, the luteal phase lasts from day 3-13 (with day 0 being the day of oestrus or 'heat'). The luteal phase is characterized by elevated concentrations of peripheral plasma progesterone, which are minimal on day 3 (< 0.5 ng/ml), remain elevated between days 6-12 (at approximately 2-3 ng/ml) and fall to 0.2 ng/ml from day 15 (Edgar and Ronaldson, 1958; Cunningham, Symons and Saba, 1975; Quirke, Bradford, Famula and Torrell, 1985). This low basal concentration of progesterone is thought to be of

adrenal origin (Robertson, 1977). These characteristic fluctuations in peripheral progesterone reflect the secretory activity from the maturing, and then demising, corpus luteum. Newly formed corpora lutea range from 8-13mm in diameter, and pink in colour, but are considered mature between days 5-14 after the beginning of oestrus. In contrast, regressing corpora lutea are white in colour, range from 2.5-10mm in diameter, and are found from days 14-26 after the beginning of oestrus (Warbitton, 1934).

In common with other prolific breeds of sheep, such as Finnish Landrace (Webb and Gauld, 1985a), Booroola Merinos are known to have follicles which ovulate at a smaller size compared to breeds of lower prolificacy (Scaramuzzi, Turnbull, Downing and Bindon, 1981). This trend of smaller preovulatory follicles in breeds of high prolificacy is not always found, for instance in Romanov ewes compared to the lesser prolific Ile-de-France ewes (Cahill and Mauléon, 1980). As a result of smaller preovulatory follicles, mean size of corpora lutea is also smaller in carriers of the Booroola fecundity (Fec^B) gene compared to non-carriers of the gene. Luteal plasma progesterone levels in Booroola Merino ewes have been shown to be independent of ovulation rate (Bindon, Cummins, Piper and O'Shea, 1981).

Ovarian progesterone is known to have an important priming effect on the uterus to allow subsequent secretion of uterine prostaglandin $F2\alpha$ ($PGF2\alpha$; Baird, Land, Scaramuzzi and Wheeler, 1976a). $PGF2\alpha$ acts as a luteolysin by causing the cessation of progesterone secretion and ultimately, structural and functional regression of the corpus luteum (sheep: McCracken, Glew and Scaramuzzi, 1970; Wilson, Cenedella, Butcher and Inskeep, 1972; Thorburn, Cox, Currie, Restall and Schneider, 1973; Baird *et al.*, 1976a; Baird, 1978a; see Hansel, Concannon and Lukaszewska, 1973; Henderson and McNatty, 1975; Horton and Poyser, 1976 for review). Active

immunization against PGF2 α is effective in blocking ovulation, but not in inhibiting either oestrus behaviour or formation of persistent luteal tissue (Bettencourt, Moffatt and Keisler, 1993). Progesterone may act to control prostaglandin release and the regulation of oxytocin receptors through different, as yet not fully understood, mechanisms (Lau, Kerton, Gow and Fairclough, 1992).

PGF2 α leaves the uterus in the uterine vein and is transferred by a counter-current exchange mechanism through the wall of the utero-ovarian vein into the ovarian artery. A rise in PGF2 α secretion from the uterus is detected by day 12-13 of the oestrous cycle, causing a decline in progesterone 24-48 hours later (Barrett, Blockey, Brown, Cumming, Goding, Mole and Obst, 1971; McCracken, Baird and Goding, 1971; Staples and Whyllie, 1985). The decline in progesterone stimulates a further release of PGF2 α from the uterus and a cascade of events proceeds until plasma progesterone concentrations are at a minimum. PGF2 α is thought to cause a decline in progesterone by inhibition of the biosynthetic pathway involved in its production (Henderson and McNatty, 1975).

Oxytocin can be detected in the preovulatory follicle during the LH surge and as the corpus luteum develops the concentration of oxytocin reaches a maximum between days 4-10 after oestrus, before declining rapidly between day 12 and the following oestrus (Webb, Mitchell, Falconer and Robinson, 1981; Flint and Sheldrick, 1982; Wathes and Swann, 1982; Sheldrick, 1991; see Wathes and Denning-Kendall, 1992 for review). Luteal oxytocin is thought to stimulate endometrial secretion of PGF2 α (Flint and Sheldrick, 1983) which, in turn, feeds back positively to stimulate ovarian secretion of oxytocin (Flint and Sheldrick, 1982; Moore, Choy, Elliot and Watkins, 1986). The link between oxytocin and luteolysis is shown by the delay in luteal regression in ewes immunized against oxytocin (Sheldrick,

Mitchell and Flint, 1980), or by continuous infusion of oxytocin (Sheldrick, 1992).

Intermittent surges of PGF2 α cause oxytocin receptor concentrations in the ovine uterus to increase to a peak at oestrus, both coinciding with the decline in peripheral progesterone concentration (Roberts, McCracken, Gavagan and Soloff, 1976; Sheldrick and Flint, 1985). It may be possible that either oxytocin and/or oestrogen stimulate the pulsatile mode of PGF2 α release such that PGF2 α reaches maximum pulsatile release around the onset of luteolysis (Zhang, Weston and Hixon, 1991; Vallet, Lamming and Batten, 1990; Lau *et al.*, 1992). Insulin-like Growth Factor-1 (IGF-1) and insulin, stimulate oxytocin release *in vitro* in both non-luteinized and luteinized bovine granulosa cells, an effect which is reduced in the presence of PGF2 α , providing evidence for paracrine regulation (McArdle and Holtorf, 1989; McArdle, Kohl, Rieger, Gröner and Wehrenberg, 1991).

Peripheral oestradiol levels peak between day 2-4 of the oestrous cycle (Holst, Braden and Mattner, 1972; Hauger, Karsch and Foster, 1977) and then fluctuate. The fluctuation in peripheral oestradiol concentrations are thought to be indicative of follicular development (Scaramuzzi and Land, 1978). In ewes, oestradiol induces oxytocin receptors after the withdrawal of progesterone such that oxytocin receptor concentrations are highest at oestrus (Sheldrick and Flint, 1985). During the luteal phase, oestradiol and progesterone act together to down-regulate the uterine oxytocin receptor (Zhang, Weston and Hixon, 1992).

The luteal phase is characterized by low frequency, high amplitude LH pulses, compared with those observed during the follicular phase. The difference in LH pulse frequency and amplitude during these two phases of

the oestrous cycle are as a result of the inhibitory action of progesterone, such that by mid-late luteal phase, LH levels are minimal when progesterone is maximal (Hauger *et al.*, 1977; Goodman and Karsch, 1980; Goodman, Pickover and Karsch, 1981; Martin, Scaramuzzi and Henstridge, 1983). Oestradiol seems to sensitize the hypothalamus to progesterone and thus further intensifies the inhibitory effect of progesterone on LH production (Baird, Swanston and Scaramuzzi, 1976b; Goodman, Legan, Ryan, Foster and Karsch, 1980). Progesterone alone does not influence the secretion of LH, or FSH, by a direct effect on the pituitary gland, although the presence of progesterone potentiates the oestrogen feedback effect on LH (Clarke and Cummins, 1984). Plasma FSH profiles, which are not pulsatile in the ewe, do vary throughout the luteal phase, although the fluctuations of FSH show no consistent trend at this time and are not thought to be involved in the determination of the length of the luteal phase (Wallace and McNeilly, 1986).

Serum concentrations of prolactin, which remain low and without significant variation during the luteal phase, increase after luteal regression and then remain elevated (Moore *et al.*, 1986). The elevated prolactin concentrations after luteal regression during the oestrous cycle probably occur as a result of elevated oestradiol concentrations during this time. Oestradiol has been shown to be stimulatory on prolactin secretion in seasonally anoestrus ewes and a single injection of oestradiol administered to ovariectomized ewes also increases prolactin concentrations (Clarke, Funder and Findlay, 1982). Care however must be exercised when studying the effects of oestradiol upon prolactin secretion since the latter is dependent upon photoperiod (see Kennaway, Dunstan and Staples, 1987 for review). The potential role of prolactin in the sheep has yet to be fully understood, although there is general consensus that prolactin is not

luteotrophic in the ewe (see Niswender, Schwall, Fitz, Farin and Sawyer, 1982 for review). Prolactin is known to cause luteal regression in mice (Grandison and Meites, 1972) and rats (Malven and Sawyer, 1966) and inhibits progesterone production in combination with LH (or its biological analogue, human chorionic gonadotrophin, hCG) in goat granulosa cell cultures *in vitro* (Mohini and Chapekar, 1983). In the ewe, prolactin production is probably mediated through the action of oxytocin, particularly in cases of prolonged luteal function (Sheldrick and Flint, 1990).

1.2.3. Temporal characteristics of the follicular phase

The follicular phase is characterized by two major events, namely oestrus and ovulation. The end of the luteal phase is associated with luteal regression and a decrease in progesterone concentration. It is this decrease in peripheral progesterone concentration that enables LH concentrations to increase (Goodman and Karsch, 1980). Approximately 60 hours after the onset of luteal regression, LH concentrations rise (Cunningham *et al.*, 1975). The rise in LH concentration lasts 48-72 hours during the follicular phase and is thought to be crucial in driving the rise in serum oestradiol which induces the LH surge and oestrus (Baird, 1978b; Karsch, Foster, Legan, Ryan and Peter, 1979).

The increasing oestradiol secretion has four important effects: it stimulates further PGF2 α secretion; it suppresses FSH secretion 48 hours prior to the preovulatory gonadotrophin surge; it induces oestrus behaviour and it elicits the surge release of LH and FSH (termed the 'preovulatory gonadotrophin surge') through a positive feedback mechanism (Goding *et al.*, 1969). At day 0, the pulsatile discharges of LH in the ewe occurs frequently (every 45 minutes) as a consequence of the

action of oestradiol which acts to limit the LH pulse amplitude but increases LH pulse frequency in ewes (Karsch, Foster, Bittman and Goodman, 1983).

After the initial stimulation of oestradiol and androgen by LH, paradoxically the preovulatory LH surge causes marked steroid inhibition, such that the secretion of oestradiol, androstenedione and testosterone are minimal at ovulation (Moor, Hay and Seamark, 1975; Thomas, Martin, Ford, Moore, Campbell and Lindsay, 1988). The paradoxical effect of LH upon oestradiol secretion is probably mediated through the increase in LH pulse frequency prior to the LH surge itself (see Karsch, 1984 for review). This dual effect of LH upon oestradiol secretion during the oestrous cycle is termed the 'biphasic response'. The LH surge is 8-12 hours in duration (Cunningham *et al.*, 1975; Goding *et al.*, 1969, Legan and Karsch, 1979) and ovulation occurs approximately 24 hours after its onset (Cumming, Brown, Blockey, Winfield, Baxter and Goding, 1971).

The timing, frequency and amplitude of FSH fluctuations during the oestrous cycle is highly variable between ewes (Miller, Nordheim and Ginther, 1981). Some authors have suggested a five day endogenous rhythm of FSH closely correlated with 'waves' of follicular growth (Bister and Paquay, 1983), whilst other authors find no evidence of this (Miller *et al.*, 1981). There is general agreement that FSH concentrations decline 24 hours preceding the preovulatory FSH and LH discharges (Wallace and McNeilly, 1986). While the physiological role of this effect has yet to be fully elucidated, it may cause a differential response in the development of ovarian follicles.

FSH concentrations on the day of oestrus have been found to be elevated in some studies (Cunningham *et al.*, 1975), but not in others (Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short and Young lai,

1972). Most authors agree that in the sheep there is a discharge of FSH coincident with the preovulatory LH discharge and that this is followed 24 hours later by a further FSH discharge at a time when LH concentrations remain low (see Goodman, Bittman, Foster and Karsch, 1981 for review). Although the function of this second FSH peak (Salmonson, Jonas, Burger, Buckmaster, Chamley, Cumming, Findlay and Goding, 1973) is unknown, it may play some role in the recruitment of ovine ovarian follicles (Cahill, Mariana and Mauléon, 1979), since in the rat it determines the number of follicles available for ovulation at the next oestrus (Sheda-Ranic and Mouldgal, 1977). Recruitment is defined as the first differentiating step towards a follicle becoming a candidate for ovulation (Tsonis, Cahill, Carson and Findlay, 1984) and is ambiguously used for either follicles at the primordial stage or at the antral stage of development (Goodman and Hodgen, 1983). In the ewe, the second FSH peak may be as a result of a change in the sensitivity of the pituitary gland to GnRH from the hypothalamus (Dobson and Ward, 1977; Narayana and Dobson, 1979), or due to the very low levels of oestradiol and inhibin secretion (Baird, Campbell, Mann and McNeilly, 1991). The secretion of FSH, but not LH, is inhibited by the synergistic negative feedback action of oestradiol and inhibin which act at the level of the pituitary gland (see Grady and Schwartz, 1981 for review; Martin, Price, Thiéry and Webb, 1988; Mann, Campbell, McNeilly and Baird, 1990). At all stages of the oestrous cycle there is a negative correlation between the secretion of oestradiol and the concentration of FSH (Baird *et al.*, 1991). After the second FSH peak, the concentration of FSH declines gradually until day 4 or 5 of the ovine oestrous cycle.

Unlike oestradiol negative feedback (Goodman and Karsch, 1981), there does not appear to be an influence of season on the ability of inhibin to suppress FSH secretion (Findlay, Gill and Doughton, 1985; see Findlay and

Clarke, 1987; Findlay, 1993 for review). Ovine inhibin was purified from follicular fluid in 1987 (Leversha, Robertson, de Vos, Morgan, Hearn, Wettenhall, Findlay, Burger and de Kretser, 1987) and is a glycoprotein hormone consisting of two dissimilar, disulphide-linked subunits (see Burger, 1989 for review). The secretion of inhibin is episodic but peripheral concentrations do not appear to be temporarily related to LH, oestradiol or FSH (Campbell, Mann, McNeilly and Baird, 1990a; McNeilly, Picton, Campbell and Baird, 1991; Campbell, Picton, McNeilly and Baird, 1991b). Throughout the oestrous cycle, FSH is regulated by negative feedback mechanisms involving inhibin (Martin, Wallace, Taylor, Fraser, Tsonis and McNeilly, 1986; Martin *et al.*, 1988; Campbell *et al.*, 1990a; Findlay, Clarke and Robertson, 1990; Baird *et al.*, 1991; see Findlay, Robertson, Clarke, Klein, Doughton, Xiao, Russell and Shukovski, 1992 for review). Bovine follicular fluid, a rich source of inhibin, can elicit a suppression of FSH when administered to inhibin-immunized seasonally anoestrus ewes (Knight, Wrathall, Glencross and McLeod, 1991).

In addition to FSH, IGF-1 also stimulates inhibin production, whilst Epidermal Growth Factor (EGF) and GnRH are inhibitory in rats (Bicsak, Ling and DePaolo, 1991). Interestingly in macaques, inhibin production by either luteinized or non-luteinized granulosa cells is stimulated *in vitro* by PGE₂ implying that locally produced prostaglandins play a role in the synthesis of inhibin in developing follicle and/or corpus luteum (Brannian, Stouffer, Molsines, Aladin, Sarkissian and Dahl, 1992). Important species differences exist however, since the corpus luteum of pigs and sheep do not contain inhibin (Bramley, Menzies, Baxter, Webb and McNeilly, 1992).

In ewes carrying the Booroola fecundity, Fec^B, gene, LH pulse frequency and amplitude during the follicular phase of the oestrous cycle

does not differ between genotypes (Scaramuzzi and Radford, 1983), although McNatty, Hudson, Henderson, Gibb, Morrison, Ball and Smith, (1987) found a consistent correlation between LH concentration and Booroola genotype. The timing of the preovulatory LH discharge may be important in prolific sheep since some of these breeds have LH discharges which occur significantly later after the onset of oestrus and luteolysis, compared to non-prolific breeds (Bindon, Piper and Thimonier, 1984; Bindon, 1984). The Booroola is one of the prolific breeds in which the LH discharge is similar to control Merinos, occurring 4.5 hours after the onset of oestrus (Bindon *et al.*, 1984; Bindon, 1984). Experiments where LH pulse frequency has been altered during the follicular phase, have shown that ovulation rate is independent of the amount and secretory pattern of LH in the preovulatory period (McNatty, Gibb, Dobson and Thurley, 1981a).

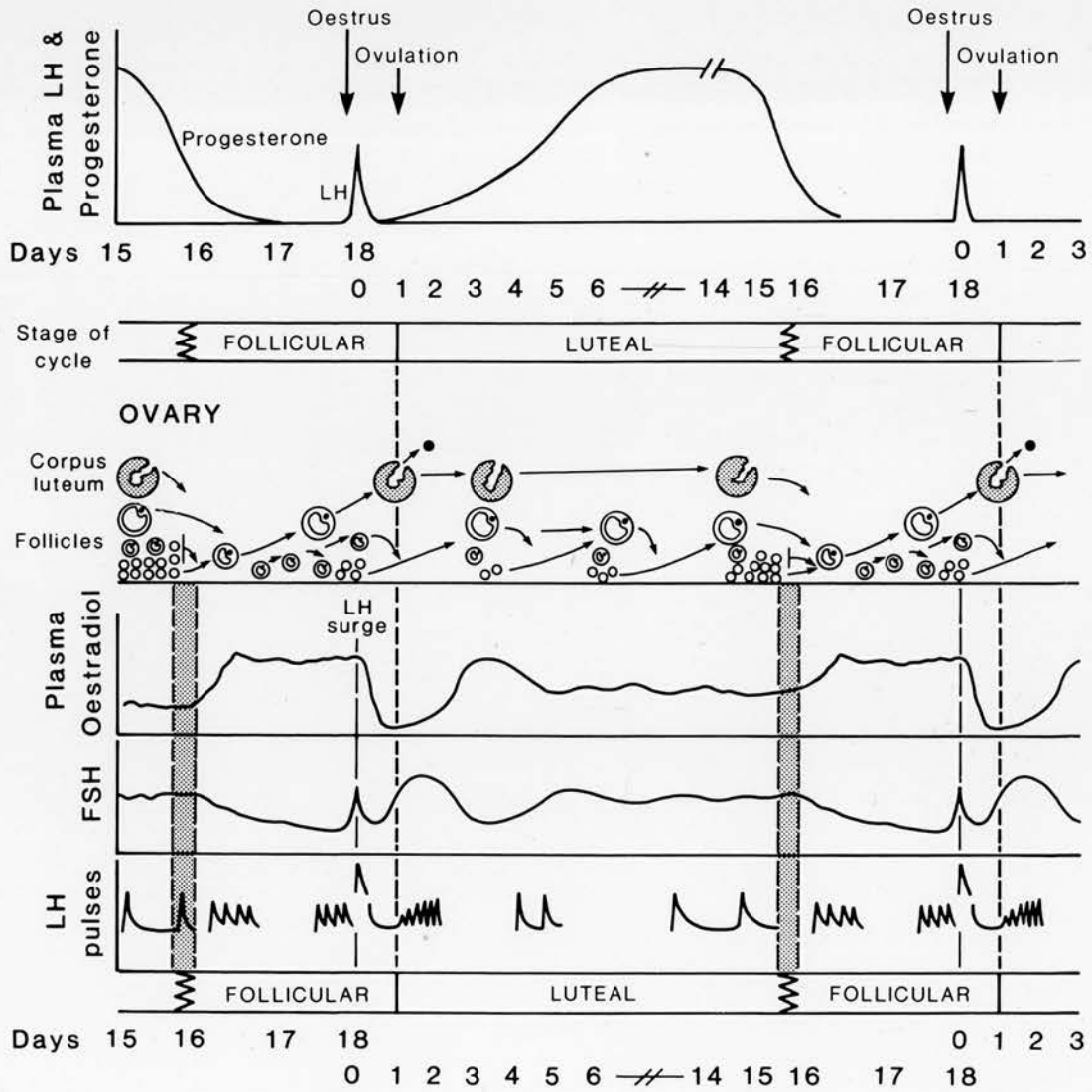
Schematic representations of the changes in plasma concentrations of LH, FSH, oestradiol and progesterone in relation to the stage of the oestrous cycle and growth of follicles in the ovine ovary are shown in **Figure 1.1**.

1.3. The gonadotrophins

1.3.1. Isolation and characterization

'Gonadotrophins' is a collective noun which includes LH and FSH and placental hormone, chorionic gonadotrophin (CG). Both a stimulating and luteinizing effect on follicles was exhibited when sheep pituitary gland extracts were given to hypophysectomized rats and rabbits (Evans 1924, cited by Parkes, 1929; Zondek and Ascheim, 1925; Evans, Simpson, Tolksdorf and Jensen, 1939). Two apparently separate gonad-stimulating hormones were subsequently detected in human pituitary samples (Fevold, Hisaw, Hellbaum and Hertz, 1933) and, for the female, the active principles were named as FSH and LH.

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The gonadotrophins are three structurally related glycoprotein hormones of approximately 40kDa in molecular weight. Gonadotrophins consist of two non-covalently linked subunits, designated alpha (α) and beta (β) (Cambarnous, 1988; Gray, 1988). The α -subunit is common to each of the hormones and the β -subunit is specific for each hormone, conferring the immunological and biological specificity (Pierce and Parsons, 1981). Beginning in the early 1970's, the primary amino acid sequences of the gonadotrophins were determined (Papkoff, Sairam, Fame and Li, 1973) and both the α and β chains were found to be highly conserved both within and between species (Sairam, 1983). Although all subunits of gonadotrophins are believed to be derived from a common ancestral gene, each subunit is encoded for by separate genes (see Hsueh, Bicsak, Jia, Dahl, Fauser, Galway, Czekala, Pavlou, Papkoff, Keene and Boime, 1989 for review).

It is now apparent that LH and FSH, from the pituitary gland or in the plasma, exist as multiple isoforms, as opposed to discrete molecular species and these isoforms differ in biological potency. The knowledge of how the concentrations of these isoforms vary in the sheep and their interaction with hormones and/or growth factors is at present unknown, but may be important in understanding the regulatory mechanisms involved in the control of reproductive function. The isoforms of gonadotrophins exhibit differences in their circulating half-life in rats (Blum and Gupta, 1985) and humans (Wide, 1986) and may be dependent on endocrine status, age and gender. FSH was first isolated from sheep pituitary glands in 1949 (Li, Simpson and Evans) and found to have a half-life of 102 minutes in the intact animal and 120 minutes in the ovariectomized sheep (Robertson, Foulds, Fry, Cummins and Clarke, 1991),

whereas the half-life of LH remains similar at 28 and 24 minutes respectively (Akbar, Nett and Niswender, 1974).

Two standard techniques are commonly used to measure gonadotrophins; either bioassays (*in vitro* or *in vivo*), or radioimmunoassay (see Wilson, Leigh and Chapman, 1990 for review). Evans *et al.* (1939), was the first to develop an FSH bioassay. This *in vivo* assay was used to compare the biological effects of different gonadotrophin preparations in a quantitative manner and was based upon stimulating growth of healthy ovarian follicles after subcutaneous injection of pituitary extracts in hypophysectomized rats. The most widely used *in vivo* bioassay for measuring FSH activity in pituitary extracts and urine was based on the augmentation of ovarian weight, developed by Steelman and Pohley (1953). Initially, LH activity was measured by the ovarian ascorbic acid depletion bioassay of Parlow (1961). One of the earliest *in vitro* bioassays involved measurement of different biological activities of FSH in various target tissues including the uptake of radiolabelled thymidine into mouse ovaries (Ryle, Cahplan, Gray and Kennedy, 1970). Most of the data regarding the concentrations of gonadotrophins has been collated by use of the radioimmunoassay technique. It is important however to bear in mind that the radioimmunoassay technique is based on the immunological, not biological, property of the hormone.

In the Booroola ewe, pituitary content of FSH has been reported to be significantly higher than for control ewes (Robertson, Ellis, Foulds, Findlay and Bindon, 1984), whilst other authors report no such difference (McNatty, Hudson, Shaw, Condell, Ball, Seah and Clarke, 1991). No genotypic difference exists in pituitary LH content for ewes with or without the Booroola fecundity gene (Robertson *et al.*, 1984). For ewes possessing the Booroola fecundity gene, Fec^B , the circulating half-life of FSH does not

differ between genotypes and is not related to the high ovulation rate observed in these sheep (Fry, Cahill, Cummins, Bindon, Piper and Clarke, 1987). At present, any possible genotypic differences in absolute amounts, or in relative proportions, of the different subunits of LH and/or FSH are unknown. Recently, the *Fec^B* mutation was found not to lie within the α and β subunit genes encoding for FSH (Montgomery, Penty, Sise and Tou, 1992b).

1.3.2. Central (hypothalamic-pituitary) control of gonadotrophin secretion

The existence of a hypothalamic factor that specifically released LH was demonstrated by McCann, Taleisnik and Friedman, (1960). This hypothalamic factor was known to be released as a result of a neural stimulus and transported to the pituitary gland by a discrete blood supply (Green and Harris, 1946), since hypothalamic lesions (Clegg and Ganong, 1960; Schneider, Crichton and McCann, 1969), or neurotransmission-blocking agents (Hansel and Trimberger, 1951), prevented ovulation. The hypothalamic factor was isolated from porcine and ovine hypothalami (Matsumo, Arimura, Nair and Schally, 1971; Schally, Arimura, Baba, Nair, Matsuo, Redding and Debeljuk, 1971a) and shown to be a decapeptide (Matsumo *et al.*, 1971). The decapeptide has subsequently been shown to be common to all mammalian species and is probably the sole neurohormone controlling the secretion of both FSH and LH from the pituitary gland (Clarke and Cummins, 1984; Karsch, 1987). For this reason, it is commonly called GnRH. GnRH administration has also been reported to cause LH and FSH release from ovine pituitary cells *in vivo* and *in vitro* (McIntosh and McIntosh, 1986; Nett, Crowder, Moss and Duello, 1981).

Reproductive function requires continuous GnRH output and early experiments infusing hypothalamic extracts into late-anoestrus, but not mid-anoestrus, sheep elicited follicle development and ovulation and also indicated a seasonal difference in the sensitivity of the adenohipophysis (Dománski and Kochman, 1968). Follicle development and ovulation can be prevented, or delayed, by active or passive immunization against GnRH (Fraser and McNeilly, 1982; McNeilly, Jonassen and Fraser, 1986; Sakurai, Adams and Adams, 1986), administration of a GnRH antagonist (Campbell, McNeilly, Picton and Baird, 1990b), or prolonged infusion of a potent GnRH agonist (McNeilly and Fraser, 1987).

In higher vertebrates, the neurones that control GnRH secretion are located within the diencephalon of the hypothalamus and their axons terminate in the median eminence and organum vasculosum of the lamina terminalis (OVLT) (Kizer, Palkovits, Tappaz, Kebabian and Brownstein, 1976; Estes, Padmanabhan and Convey, 1977). In the ewe, the first study to map GnRH localization was by Dubois and Barry (1974), although a more detailed study was subsequently published by Polkowska (1981). GnRH preparations can induce detectable releases of LH and FSH (sheep: Foster and Crighton, 1974; Lincoln, 1979; cattle: Kesner and Convey, 1982; Kittok, Britt and Convey, 1973), with a greater response being obtained just prior to the preovulatory gonadotrophin surge (sheep: Foster and Crighton, 1976; cattle: Convey, Beal, Seguin, Tannen and Lin, 1976).

GnRH is thought to bind to a single class of high affinity receptors in the pituitary cell plasma membrane (Wagner, Adams and Nett, 1979) and elicits its effect through a second messenger system involving calcium, with the aid of the calcium binding protein, calmodulin (Stern and Conn, 1981; Drouva, Laplante and Kordon, 1985). GnRH stimulates pituitary cells

to increase their phospholipid turnover which, in turn, activates protein kinase C and stimulates gonadotrophin synthesis and release (see Conn, 1986; Clayton, 1988 for review).

GnRH can stimulate both the production of gonadotrophin messenger ribonucleic acid (mRNA) and the synthesis of both subunits of LH (Hamernik, Crowder, Nilson and Nett, 1986; Starzec, Counis and Jutisz, 1986; Yuan, Swerdloff and Bhasin, 1988) and FSH (Kalra and Kalra, 1985; Mercer, Clements, Funder and Clarke, 1989). LH and FSH are known to act at the level of the anterior pituitary gland in an ultra-shortloop feedback, or autoregulatory way, to inhibit further production of LH or FSH respectively (Patriitti-Laborde, Yoshimoto, Wolfson and Odell, 1979; Patriitti-Laborde, Wolfson and Odell, 1981). Disconnection of the hypothalamus from the pituitary gland in sheep leads to decreased pituitary responsiveness to exogenous GnRH, implying a reduction in GnRH receptor number and/or a decrease in LH synthesis (Clarke, Cummins, Findlay, Burman and Doughton, 1984). Further evidence that GnRH is required for the induction of its own receptor was shown in rats by active immunization of GnRH, which decreased GnRH pituitary receptor number (Popkin and Fraser, 1985). Inhibin, alone, or in conjunction with oestradiol has also been shown to increase the number of GnRH receptors in sheep (Gregg, Schwall and Nett, 1991).

The biological importance of GnRH pulse release (see Marshall, Dalkin, Haisenleder, Paul, Ortolano and Kelch, 1991 for review) has been investigated by comparison of either pulsatile or continuous GnRH infusion (see Haresign, Foxcroft and Lamming, 1983 for review). GnRH pulse frequency regulates the synthesis of both the α and β -subunits of LH (Haisenleder, Khoury, Zmeilu, Papavasiliou, Ortolano, Dee, Duncan and Marshall, 1986; Leung, Kaynard, Negrini, Kim, Maurer and Landefeld,

1987). Constant GnRH stimulation has been found to be necessary for gonadotrophin release during the preovulatory surge in sheep (Webb, England and Fitzpatrick, 1981; Nett, Crowder and Wise, 1984). Continuous infusion of GnRH into either ovariectomised ewes (Nett, Crowder, Moss and Duello, 1982; Crowder, Herring and Nett, 1986), or seasonally anoestrous ewes (Reeves, Tarnavsky and Chakraborty, 1974; Amundson and Wheaton, 1979), caused an initial surge in pituitary gonadotrophin release, but concentrations declined to baseline, even though infusion was continued. The decline in pituitary gonadotrophin release is due to 'down-regulation' of the receptors and desensitization (Khalid, Haresign and Hunter, 1991). Clayton (1982) observed that infusion of low doses of GnRH into rats, as might be expected to occur *in vivo* between pulses, caused 'up-regulation' of pituitary LH receptors and primed the pituitary for each GnRH pulse. Further studies in sheep have shown that GnRH pulse amplitude is not an important influence on the rate of pituitary desensitization compared to pulse frequency. GnRH pulses which were less than 16 minutes apart actually increased the rate of desensitization as the LH release mechanism in pituitary cells needed time to recover sensitivity between stimulations (McIntosh and McIntosh, 1983).

When the frequency of pulsatile secretion is low, such as occurs during seasonal anoestrus or in the luteal phase, GnRH normally affects LH pulsatility on a one-to-one basis in sheep (Clarke and Cummins, 1982; Levine, Kwok-Yuen, Pau, Ramirez and Jackson, 1982; Barrell *et al.*, 1992). A greater disassociation between GnRH and LH pulses can be observed in sheep when GnRH pulse frequency is high, such as occurs during the follicular phase of the oestrous cycle (Barrell *et al.*, 1992). Physical removal of the GnRH stimulus by hypothalamic-pituitary disconnection (HPD) caused a significant decrease in plasma LH concentrations in ewes

(Clarke, Cummins and de Kretser, 1983), but not in sows (Kraeling, Kesner, Estienne, Estienne, Barb and Rampacek, 1990). An ovariectomized-hypothalamo-pituitary disconnection (HYDOVX) animal is deficient in endogenous GnRH and devoid of ovarian hormones, but with a functionally intact pituitary gland. Experimental use of HPDOVX ewes have shown that GnRH pulse frequency may be important in controlling LH pulse frequency and GnRH pulse amplitude in controlling LH pulse amplitude (Clarke *et al.*, 1984). Other studies using HPDOVX ewes have suggested that GnRH pulse frequency could account for both LH pulse frequency and amplitude (Clarke and Cummins, 1985) according to the 'two pool pituitary LH storage theory' (Yen and Lein, 1976; Stelmasiak and Cumming, 1977).

The two pool pituitary LH storage theory is based on the biphasic LH response to high-doses of GnRH. The first phase of this response demonstrates the size of the 'readily-releasable' pool of LH, whilst the second phase response depends on mobilization of pituitary LH from a 'releasable pool' to the readily-releasable pool, or the synthesis of new hormone (humans: Bremner and Paulsen, 1974, sheep: Bremner, Findlay, Cummings, Hudson and de Kretser, 1976). The existence of two pools of LH explains the biphasic response that can be seen following a GnRH challenge. In response to a low dose of GnRH, LH is released from the readily releasable pool and if the dose of GnRH is greater than the LH stored in the readily releasable pool, LH is discharged from the releasable pool (Bremner *et al.*, 1974). The size of the readily-releasable pool may be regulated by the frequency with which it is emptied (ie. by GnRH pulse frequency), thus GnRH pulse frequency may determine LH pulse amplitude as a function of the size of the releasable pool (Clarke and Cummins, 1985). Recent evidence has suggested that, following repeated stimulation with GnRH, LH synthesis is stimulated, and this newly synthesized LH is

preferentially released, irrespective of the presence or absence of the readily releasable and releasable pools of stored LH (Evans, 1991).

In sheep, frequent sampling of hypophyseal portal blood has been used to characterize both the positive and negative feedback control of GnRH secretion. Prior to the preovulatory LH surge, the frequency and amplitude of GnRH pulses are reduced, due to the negative feedback effect exerted by oestradiol. GnRH pulse frequency and amplitude subsequently increases through the positive feedback effect of oestradiol inducing the release of gonadotrophins (Clarke, Cummins, Jenkin and Phillips, 1989; Caraty, Moenter, Locatelli, Martin and Karsch, 1990; Moenter, Caraty, Locatelli and Karsch, 1991). However, during the luteal phase, high circulating concentrations of progesterone suppress the frequency of pulsatile GnRH and oestradiol release and therefore indirectly depresses LH secretion (see Karsch, 1987 for review).

Use of GnRH agonists (rat: Berardo and DePaolo, 1986; hamster: Chappel, Miller and Hyland, 1984), or antagonists (rat: Grady, Shin, Charlesworth, Cohen-Becker, Smith, Rivier, Rivier, Vale and Schwartz, 1985; monkey: Fraser, Abbott, Laird, McNeilly, Nestor and Vickory, 1986; sheep: Condon, Heber, Stewart and Sawyer, 1984) have shown a differential effect of GnRH on LH and FSH, such that LH is preferentially affected compared to FSH. In the sheep, each GnRH pulse does not produce a pulse of FSH (see Clarke, 1989 for review). Accumulating evidence suggests that whilst GnRH may be necessary for FSH synthesis, it plays a minor role in the regulation of FSH release (see Price 1991 for review).

Various studies where ewes have been immunized against GnRH have always decreased LH release, but FSH has decreased (Adams and Adams, 1986), increased (Fraser and McNeilly, 1983), or remained

unaffected (Clarke, Fraser and McNeilly, 1978; Lincoln and Fraser, 1979). These discrepancies in the release of FSH following GnRH manipulation supported the suggestion of the existence of separate releasing hormones for each pituitary gonadotrophin. An alternative explanation for the discrepancies in FSH release following GnRH manipulation may be due to differences between the studies in the time interval elapsed after immunization before FSH was measured. FSH concentrations take 2-3 weeks to decline whereas LH declines rapidly after immunoneutralization of GnRH suggesting differences between the gonadotrophins in their synthesis and storage (McNeilly *et al.*, 1986; McNeilly, Evans, Bramley, Brown, Clark and Webb, 1993). However, comparisons of the biopotency of various hypothalamic extracts with purified GnRH did support the hypothesis for the existence of a specific FSH-releasing hormone (Currie, Johannsson, Folkers and Bowers, 1973; Johannsson, Currie, Folkers and Bowers, 1973). This hypothesis was strengthened by the discovery of FSH-releasing activity in hypothalamic extracts, which were essentially free of LHRH activity (Bowers, Currie, Johannsson and Folkers, 1973; Mizunuma, Samson, Lumpkin and McCann, 1983). However, this FSH-releasing agent has never been isolated (Schally *et al.*, 1971a; Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk and White, 1971b; Schally, Arimura, Redding, Debeljuk, Carter, Dupont and Vilchez-Martinez, 1976). Furthermore, recent evidence has shown that by manipulating the characteristics of GnRH pulse amplitude, frequency and duration, a divergent release of LH and FSH from pituitary cells can be obtained *in vitro* (Kellom and O'Connor, 1991). It may be that no such specific FSH-releasing hormone exists and that LH is acutely regulated by GnRH secretion, whereas FSH is chronically controlled by GnRH secretion (Martin *et al.*, 1986).

Sawyer, Markee and Hollinshead, (1947) suggested that catecholamines (norepinephrin and dopamine) could play a role in the control of gonadotrophin release in rabbits. A low concentration of noradrenaline decreased LH pulse frequency when infused into the medial preoptic area of ovariectomized, conscious rats (Leipheimer and Gallo, 1985; Condon, Handa, Gorski, Sawyer and Whitmoyer, 1986). Catecholamines inhibit LH release in the ewe by suppressing GnRH pulse frequency (Havern, Whisnant and Goodman, 1991) and dopamine receptor stimulants markedly decrease pulsatile LH release in ovariectomized rats (Drouva and Gallo, 1976). Adrenergic blocking agents decrease LH release in the ovariectomized animal (ewe: Jackson, 1977; rat: Weick and Noh, 1984; hamster: Chappel *et al.*, 1984), whilst dopamine antagonists have been reported to increase LH and, to a lesser extent, FSH secretion in ovariectomized hamsters (Chappel *et al.*, 1984). The catecholamines are thought to modify the responsiveness of animals to the negative feedback of oestrogen (see Spicer, 1986; Thiéry and Martin 1991 for review). Neurones using opioid peptide transmitters (β -endorphin and enkephalin) are generally inhibitory to gonadotrophin secretion (Drouva, Epelbaum, Tapia-Arancibia, Laplante and Kordon, 1981).

To add a further degree of complexity to the regulation of FSH and LH release from the pituitary gland, some authors have postulated the existence of a shortloop feedback mechanism from the pituitary gland to the hypothalamus, and an ultra-short loop feedback from within the pituitary gland to control gonadotrophin secretion. The control of FSH release by FSH (rabbit: Patritti *et al.*, 1981; rat: Ojeda and Ramming, 1969), LH control by LH (Ojeda and Ramming, 1969) and GnRH control by GnRH (Hyyppa, Motta and Martini, 1971; DePaolo, King and Carrillo, 1987; Zanisi, Messi, Motta and Martini, 1987) have been reported.

No Booroola gene specific differences have been found in the concentration of GnRH in hypothalamic or extra-hypothalamic regions of the brain (Gale, Smith and McNatty, 1988). Although the pattern of GnRH secretion differed (McNatty, *et al.*, 1987), no differences in GnRH-receptor binding to pituitary tissue has been found (Fleming, Lun, Smith and McNatty, 1990). Studies using HYDOVX ewes possessing the Booroola fecundity gene have shown that GnRH preferentially stimulates FSH, but not LH, in carriers compared to non-carriers of the gene (McNatty *et al.*, 1991).

The central control of gonadotrophin secretion in the ewe is complex and is not only dependent on the interaction between the neural and hormonal signals originating from the hypothalamus and pituitary gland, but also upon other physiological influences, for example, hormonal messages from the ovaries and the mechanisms which in turn govern them.

1.3.3. Actions of gonadotrophins

The stimulating effects of gonadotrophins on reproduction have been known for some time (Fevold *et al.*, 1933; Evans *et al.*, 1939). Gonadotrophins specifically target certain cells within the ovarian follicle to control folliculogenesis (Hisaw, 1947). Folliculogenesis is a process involving follicle growth and development from a pool of primordial follicles through the preantral and antral phases to culminate either in atresia or ovulation (Cahill, Oldham, Cognié, Ravault and Mauléon, 1984). Gonadotrophins have also been implicated in controlling steroid synthesis and secretion by the developing follicle (termed 'steroidogenesis'). The influence of gonadotrophins on folliculogenesis and steroidogenesis will be discussed in more detail in the following sections.

1.4. Folliculogenesis

1.4.1. Characteristics

The ovary of an adult ewe contains between 12,000 and 86,000 primordial follicles and 100-400 growing follicles of which, 10-40 are visible on the ovarian surface (Cahill *et al.*, 1979). Driancourt and Fry (1988) divided folliculogenesis into 2 stages according to follicular requirements for gonadotrophins, termed 'basal' and 'tonic'. Basal folliculogenesis involves development of follicles up to 2mm in diameter and may be partially independent of gonadotrophins. Tonic folliculogenesis is totally dependent upon gonadotrophins and involves development of follicles from approximately 2mm in diameter to preovulatory size.

At the start of basal development, the initiation of follicle growth involves the passage of primordial follicles from the dormant phase to the transitory phase, before the growth phase is entered. This initiation of follicle growth may be dependent on the number of small antral follicles (Krarup, Pederson and Faber, 1969) and is known to be influenced by season and nutrition (Cahill and Mauléon, 1980; Cahill, 1981). Despite much speculation as to the mechanism driving the initiation of follicle growth, this remains one of the least understood areas of ovarian biology (Gosden, 1990a). One interesting hypothesis, the 'production hypothesis', is based on cytogenic evidence only, but states that the age at which follicles start to grow is predetermined by the order in which they start to form (Henderson and Edwards, 1968). However, it is a difficult concept to understand since the formation of follicles occurs over a much shorter period of time compared to the reproductive lifespan of the animal.

In the sheep, it has been estimated that from the start of the development of a primordial follicle to the ovulatory stage takes 6 months (Cahill, 1981). The rate of follicular growth, as determined by the doubling time in the number of granulosa cells, is slow at first until the follicle reaches 0.4mm in diameter (Turnbull, Braden and Mattner, 1977). Follicular growth then accelerates to a maximum, before declining when the follicle reaches 2mm in diameter for monovulatory breeds (Turnbull, *et al.*, 1977). The increased rate of follicle growth is due to the increase in granulosa cell number and DNA synthesis (Roy and Greenwald, 1986; Wang, Roy and Greenwald, 1991). In the sheep, large antral follicles (>2mm in diameter) are 'recruited' to become candidates for ovulation (McNatty, Gibb, Dobson, Ball, Coster, Heath and Thurley, 1982; Driancourt, Gauld, Terqui and Webb, 1986; Webb, Gauld and Driancourt, 1989).

The timing of recruitment varies with species, since in rats it takes place at the time of the previous ovulation (Hirschfield and Midgley, 1978), whilst in mares it occurs during the late luteal phase (Driancourt and Palmer, 1984). In sheep, follicles are thought to be recruited soon after luteolysis (Driancourt and Cahill, 1984), since unilateral ovariectomy around the time of luteolysis (day 14), but not 48 hours after it, does not affect the number of ovulations from the remaining ovary (Land, 1973; Findlay and Cumming, 1977).

After recruitment has occurred these follicles continue their growth and maturation until the final step, whereby some of the recruited follicles are selected to ovulate instead of degenerating. The timing of selection is highly variable, but all follicles that can be clearly defined as selected are >4mm in diameter in monovulatory breeds (Driancourt, 1987; Webb *et al.*, 1989). Selected follicles are usually identified as 'dominant', that is

possessing the ability to escape follicle death, atresia, at least for a limited period. Ovulatory follicles possess a number of distinct characteristics including, increased size (monkey: Dufour, Whitmore, Ginther and Casida, 1972; Clark, Dierschke and Wolf, 1979; ewe: Driancourt and Cahill, 1984), increased oestradiol secretion (monkey: diZerega and Hogden, 1981; ewe: England, Dahmer and Webb, 1981) and the capacity of granulosa cells to bind LH (monkey: Zeleznik, Schuler and Reichert, 1981; ewe: Webb and England, 1982a). Carson, Findlay, Burger and Trounson, (1979) have shown a ten fold increase in the number of LH receptors on granulosa cells of sheep follicles which are greater than 4mm in diameter.

The mechanism of selection is poorly understood, but could be explained by two different hypothesis. The first hypothesis is the 'development stability hypothesis', whereby a mature follicle is unresponsive to changes in the hormonal milieu which causes other follicles to become atretic. The second hypothesis is the 'active elimination hypothesis', whereby one follicle of a given size actively inhibits the growth of other follicles. In monkeys (diZerega and Hodgen, 1980) and cattle (Ko, Kastelic, Del campo and Ginther, 1991) follicle dominance is achieved by active elimination, but in sheep the data suggests that active dominance is not operative (Driancourt, Webb and Fry, 1991). In the sheep, it is thought that preovulatory follicles which are destined to ovulate can do so because they have a lower 'threshold' to the decline in FSH concentrations seen at around the time of luteolysis (Henderson and McNatty, 1975; Baird 1983; Henderson, Ellen, Savage, Ball and McNatty, 1986). Thus each ovine ovarian follicle may require a different threshold, or peripheral concentration of FSH stimulation, in order to mature further and ovulate (Hillier, 1990). It has been hypothesized that a mechanism to sustain FSH concentrations around the time of luteolysis could be important

in increasing the number of selected follicles (Hudson, McNatty, Ball, Gibb, Heath, Lun, Kieboom and Henderson, 1985).

Patterns of follicular development have been characterized for rodents (see Richards, 1980 for review) and cattle (see Ireland, 1987; Fortune, Sirois, Turzillo and Lavoie, 1991 for review). In the ewe, some authors report one (Hutchinson and Robertson, 1966), two (Brand and de Jong, 1973), or three/four (Smeaton and Robertson, 1971) waves of follicular growth. Whatever the true number of follicular growth waves it is known that follicles grow and regress asynchronously throughout the oestrous cycle (Turnbull *et al.*, 1977). It is currently difficult to accept the theory of follicular growth waves in sheep because (i) normal and atretic follicles of different sizes are present on each day of the oestrous cycle, suggesting continual and asynchronous growth and regression of follicles and (ii) large diameter follicles during the luteal phase are not present in all ewes (Smeaton and Robertson, 1971; Lahlou-Kassi and Mariana, 1984). However, when it is possible to perform ultrasound studies in sheep, similar to cattle, this possible absence or presence of follicular waves will be determined.

The majority of follicles cease their growth in the antral phase (Brand and de Jong, 1973) and although morphological and functional changes induced by atresia are well documented, the mechanisms involved remain unknown (Moor, Hay, Dott and Cran, 1978; Carson *et al.*, 1979; Tsafiriri and Braw, 1984). A reduction in follicular production of testosterone may lead to a reduction in the rate of atresia (Louvét, Harman, Schreiber and Ross, 1975). Testosterone and its 5 α -reduced metabolites have been shown to inhibit the FSH-induced stimulation of LH receptors on the granulosa cells (Farookhi, 1980) and non-aromatizable 5 α -reduced androgen metabolites are thought to be potent inhibitors of granulosa cell

aromatase reaction *in vitro* (Hillier, Van Den Boogard, Reichart and Van Hall, 1980). It has been suggested that atretic follicles may serve a physiological function by providing additional steroid substrates for follicles which are destined to ovulate (Westhof, Westhof, Braendle and diZerega, 1991).

In sheep, investigations into the potential mechanisms controlling folliculogenesis, and ultimately ovulation rate, have been made by comparing characteristics in breeds that differ in prolificacy (see Bindon *et al.*, 1985; Webb *et al.*, 1991 for review). Driancourt *et al.* (1986) determined that different prolific breeds achieve high ovulation rates by different mechanisms, either by recruiting a higher number of follicles, such as in the D'Man (Lahlou-Kassi and Mariana, 1984) and Romanov (Driancourt *et al.*, 1986) breeds, or by increasing the number of selected follicles, as seen in Finnish Landrace ewes (Webb *et al.*, 1989). In ewes possessing the Booroola fecundity gene, the high ovulation rate is attained by an extended recruitment phase coupled with a low intensity of selection and the ability of follicles to 'wait' for ovulation (Driancourt, Cahill and Bindon, 1985).

In common with other prolific breeds (Romanov: Driancourt *et al.*, 1986; Finnish Landrace: Webb, Land, Pathiraja and Morris, 1984), the diameter of preovulatory follicles from Booroola ewes are smaller (Scaramuzzi and Radford, 1983), although the follicle growth rates remain unaltered (McNatty, Henderson, Lun, Heath, Ball, Hudson, Fannin, Gibb, Kieboom and Smith, 1985a; Driancourt *et al.*, 1986; Driancourt and Fry, 1988; Castonguay, Dufour, Minvielle and Estrada, 1990). As a result of the reduced diameter, it may be necessary for carriers of the Fec^B gene to provide more follicles in order to produce the same quantity of oestradiol as non-carriers (Baird, Ralph, Seamark, Amato and Bindon, 1982; McNatty, Lun, Heath, Ball,

Smith, Hudson, McDiarmid, Gibb and Henderson, 1986b).

Inhibin content of ovaries from Booroola Merino sheep has been reported to be only one third that of control strain Merino ewes (Cummins, O'Shea, Bindon, Lee and Findlay, 1983), although the results of this study may be dependent upon the stage of oestrous cycle; did not clarify which class of follicles were producing the inhibin (see Bindon, Piper, Cummins, O'Shea, Hillard, Findlay and Robertson, 1985 for review) and involved comparison of Booroola Merinos with control Merinos which were of a different strain. For sheep carrying the Booroola fecundity gene, inhibin concentrations do not seem to be related to the genotypic differences in prolificacy (Tsonis, McNeilly and Baird, 1988; McNatty, Heath, Hudson, Ball and Condell, 1992; Henderson, McNatty, Wards, Heath and Lun, 1991; McNatty *et al.*, 1992) and the inhibin deficiency may not be directly related to the function of the Booroola fecundity gene (Driancourt, Fry, Campbell and McNeilly, 1991). However, further study is required using animals of known genetic background whose genetic relationship to members within the same experimental group can be carefully regulated in order to determine the exact relationship between the presence of the Booroola fecundity gene and the relationship to inhibin.

Different follicle populations for ewes possessing the Booroola fecundity gene have been identified and compared to non-carriers. Fec^B carriers have a greater number of primordial follicles, of which more enter the growing phase, compared to primordial follicles from control ewes (Driancourt *et al.*, 1985). The results from this data, whilst supporting claims of a negative correlation between the number of primordial follicles and mean ovulation rate in different genetic lines (Land, 1970; Cahill *et al.*, 1979), must be considered with care because of the use of inappropriate control lines. The number of preantral follicles in carriers of the Booroola

fecundity gene is also higher than non-carriers, although the number of antral follicles does not differ between genotypes (Driancourt *et al.*, 1985). The lack of association between number of antral follicles and ovulation rate is found both between breeds and within prolific breeds or strains (Lahlou-Kassi and Mariana, 1984; Driancourt *et al.*, 1986). Finnish Landrace ewes achieve high ovulation rates through a marked reduction in the number of follicles lost through atresia, although no such relationship exists between Booroola genotypes (Driancourt *et al.*, 1985).

1.4.2. Role of gonadotrophins

Follicle growth from the transitory phase to the growth phase is known to be gonadotrophin dependent in mice (Carroll, Whittingham and Wood, 1991b) and in sheep (Cahill and Mauléon, 1981). The level of dependence upon gonadotrophins to initiate follicle growth may be highly dependent on species, since in rodents some authors report the process to be independent of gonadotrophins (Paesi, 1949; Peters, Byskov, Linton-Moore, Faber and Anderson, 1973). In sheep, gonadotrophins are thought to be facilitory and not obligatory for the initiation of follicle growth, since hypophysectomy resulted in a slower rate of growth of primordial follicles to the preantral stage (Dufour, Cahill and Mauléon, 1979). The gonadotrophins are also thought to be primarily responsible for controlling follicular recruitment in sheep (McNatty, Hudson, Gibb, Ball, Henderson, Heath, Lun and Kieboom, 1985b; see Driancourt, Gibson and Cahill, 1985 for review) with FSH having a priming effect on the follicles to sensitize them to the potentiating effects of LH (Gray, Cartee, Stringfellow, Riddell, Riddell and Wright, 1992). The regulating effect of gonadotrophins on follicle development is continuous from recruitment until selection.

There is at present no direct evidence that LH or FSH are actively responsible in controlling the selection process (Salmonson *et al.*, 1973; McNeilly, McNeilly, Walton and Cunningham, 1976; McNatty *et al.*, 1981a), although LH and FSH have been shown to regulate the growth of preovulatory follicles (ewe: Baird, Swanston and McNeilly, 1981, McNatty *et al.*, 1981a; Picton, Tsonis and McNeilly, 1990; Picton and McNeilly, 1991; rat: Richards, Jonasson and Kersey, 1980; human: Mais, Kazer, Cetel, Rivier, Vale and Yen, 1986; Polson, Mason, Saldahna and Franks, 1987; monkey: Zeleznik and Kubik, 1986; cow: Adams, Matteri, Kastelic, Ko and Ginther, 1992). The most notable long-term (70 days) effect of hypophysectomy in ewes is the absence of large antral follicles, adding support to gonadotrophic involvement in folliculogenesis, particularly in the later stages of development (McNatty, Heath, Hudson and Clarke, 1990). It may be that pulsatile LH secretion plays an active role in preovulatory follicle selection by altering the responsiveness of the growing follicle to FSH and may combine with the decline in FSH secretion during the follicular phase to enhance atresia in follicles deprived of FSH (McNeilly *et al.*, 1991).

1.4.3. Other factors involved

Much emphasis has been placed on gonadotrophic control of folliculogenesis (McNeilly *et al.*, 1991). However, it is important to recognise that other hormones and growth factors may regulate follicular development directly or indirectly, through interaction with gonadotrophins, (see Tonetta and di Zerega, 1989; Hunter, Biggs, Faillace and Picton, 1992 for reviews).

Oestradiol, produced by developing follicles, is a potent amplifier of FSH action in promoting folliculogenesis (Tonetta and Ireland, 1984; Tonetta, Spicer and Ireland, 1985), whilst progesterone is known to have

direct effects on follicle development (rat: Buffler and Roser, 1974; hamster: Greenwald, 1977; monkey: Goodman and Hodgen, 1977; mouse: Telfer, Gosden and Faddy, 1991). It has been suggested that prolactin can affect follicular development (McNatty, Hunter, McNeilly and Sawers, 1975) by being inhibitory to aromatase activity (rats: Wang, Hsueh and Erickson, 1980). GnRH also has an inhibitory effect on aromatase activity and progesterone secretion from rat granulosa cells *in vitro* (see Hsueh, Jones, Adashi, Wang, Zhuang and Welsh, 1983 for review). Insulin is important in maintaining follicle viability *in vitro*, although in hamster granulosa cells, insulin alone is insufficient to maintain follicle health, suggesting the requirement for other factors for follicle survival (Roy and Greenwald, 1989). An unidentified factor present in the ovine follicular fluid is known to act directly on the ovary to induce atresia of large follicles and results in immediate suppression of ovarian inhibin, oestradiol and androstenedione secretion (Campbell, Picton, Mann, McNeilly and Baird, 1991a).

The role of ovarian growth factors in the control of ovarian function has become an area of intensive research in current years and is the subject of numerous reviews (Gospodarowicz, 1983; Adashi, Resnick, D'Ecole, Svoboda and Van Wyk, 1985a; Dorrington, Bendell, Chuma and Lobb, 1987; Holly and Wass, 1989; Carson, Zhang, Hutchinson, Herington and Findlay, 1989; Sara and Hall, 1990; Hammond, Mondschein, Samaras, Smith and Hagan, 1991). Various growth regulators, such as luteinizing inhibitor and oocyte maturation inhibitor have been implicated in the control of folliculogenesis (see Hammond, 1981 for review). Ovarian growth factors have been studied extensively *in vitro* particularly with reference to their action on granulosa cell differentiation and their interaction with gonadotrophins, particularly FSH (Channing, 1970; Richards and Midgley,

1976; Dorrington and Armstrong, 1979; Hillier, 1981; Hsueh *et al.*, 1983; Roy and Greenwald, 1991a).

In many species, investigations into growth factor action upon the follicle, or component cell types within the follicle, have identified general mitogenic and developmental effects by a number of growing factors, for example: transforming growth factor alpha (TGF- α ; Roberts and Sporn, 1985; Lobb, Skinner and Dorrington, 1988; Skinner, 1989); TGF- β (Knetch and Feng, 1986); fibroblast growth factor (FGF; Gospodarowicz, Cheng, Lui, Barid, Esch and Bohlen, 1985); epidermal growth factor (EGF; Gospodarowicz and De Bialecki, 1979; Roy and Greenwald, 1991a); IGF-1 (Schams, Koll and Li, 1988; Savion, Lui, Laherty and Gospodarowicz, 1981; Webb and McBride, 1991) and platelet derived growth factor (PDGF; May, Frost and Bridge, 1990; May, Bridge, Gotcher and Gangrade, 1992).

Other growth factor effects on follicular development may be more specific. Inhibin-like TGF- β is involved in regulating aromatase activity and probably the recruitment/selection process of follicles (Ying, Becker, Ling, Veno and Guillemin, 1986b), whilst FGF is thought to be involved in the development of the rich vasculature of the theca interna (Neufeld, Ferrara, Schweigerer, Mitchell and Gospodarowicz, 1987). Data from rat and cow granulosa cell models have implicated activin in having a unique role in the acquisition and propagation of FSH receptors in preantral follicles (see Mather, Woodruff and Krummen, 1992; Findlay, 1993 for review). More examples of specific growth factor action include the inhibitory activity of EGF on follicular cell growth (Skinner, Keski-Oja, Osteen and Moses, 1987) and the possible involvement of IGF-1 in antrum formation and follicle atresia through the regulation of proteoglycans (Adashi, Resnick, Svoboda and Van Wyk, 1986a).

It is important to note that the physiological effects exerted by a growth factor will depend on the hormonal and growth factor milieu and the interaction between them. Most of the current research implicating growth factor(s) involvement in follicular development has evolved by investigating the effects of granulosa or theca cell proliferation and/or differentiation *in vitro*. Further indications that growth factors are involved in regulating follicular development has been gained by the measurement of the concentration of the growth factors within the follicular fluid, or by investigation of gene expression with the components of the follicle.

Attempts to modulate follicular development *in vivo* have led to more conflicting results, for example, EGF stimulated development in rats (Gospodarowicz, Mescher and Birdwell, 1978), but inhibited growth in mice (Lintern-Moore, Moore, Panaretto and Robertson, 1981). However, growth factor actions upon follicular development do have fairly consistent effects across species. Important species differences do exist and should not be overlooked, for example, TGF- β enhances FSH-stimulated effects upon rat granulosa cells, but is a predominantly negative regulator of growth and differentiation in cultured porcine granulosa cells (Mondschein, Canning and Hammond, 1988). More study is required to clarify the role of growth factors in controlling follicular development, particularly through the use of *in vivo* experiments or by the development of culture systems which mimic more closely *in vivo* conditions.

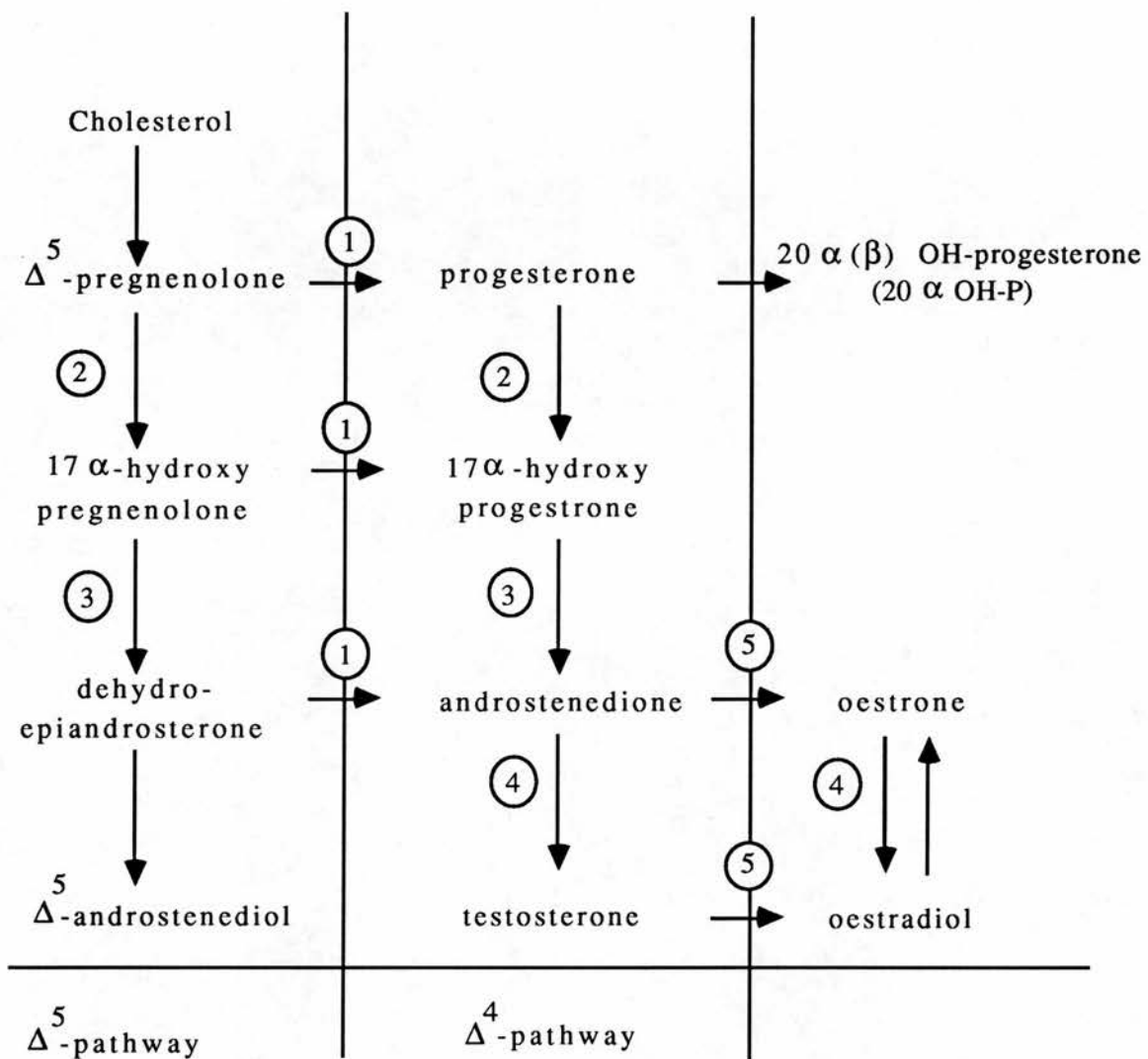
1.5. Ovarian steroid production

1.5.1. Ovarian steroid synthesis

The biochemical pathways involved in ovarian steroid production or 'steroidogenesis' are well established (see Butt, 1975 for review). The common steroid precursor is cholesterol, which in turn can be synthesized to the intermediate precursor, pregnenolone. Once pregnenolone is formed further transformations follow either the delta (Δ)⁴ or the Δ ⁵ pathway, depending on the species or tissue compartment, to produce progesterone and androstenedione, the precursor of testosterone (Ryan and Smith, 1965; Short, 1962). Using androgens, for example androstenedione and testosterone, as the main precursors, the ovary synthesizes the major oestrogens, oestradiol-17 β and oestrone (see **Figure 1.2.**). The biosynthesis of steroid hormones is complex involving many enzymatic stages and with different biosynthetic pathways leading to the same products. Some of the precursors of the biosynthetic pathway are termed 'prohormones', which, once transformed into the active substance, elicit their effect at a target tissue.

1.5.2. Ovarian steroid secretion

Ovarian stroma seems to function in different ways in different species, such that in the hamster and rabbit it is the major source of progestins (Leavitt, Bosley and Blaha, 1971), whereas in women, mares and sheep, it is thought to secrete androstenedione and some testosterone (Short, 1964; Baird and Fraser, 1974). Depending on the species, theca and granulosa cells of the follicle and the corpus luteum are the principal sites of oestrogen biosynthesis (Ryan and Short, 1965; Dorrington, Moon and Armstrong, 1975), unlike the stroma which cannot synthesize oestrogen



Major Enzymes

- ① 3β -hydroxysteroid dehydrogenase
- ② 17α -hydroxylase
- ③ C17-C20 lyase
- ④ 17β -hydroxysteroid dehydrogenase
- ⑤ Aromatase enzyme system

Modified from Hammerstein (1974)

from androgen (Channing, 1989). Progesterone is secreted by the corpus luteum of most species or, in some species, by stroma and/or granulosa cells of the preovulatory follicle (see Peters and McNatty, 1980 for review). The most impressive ovarian endocrine tissue is the follicle, which alters the emphasis of its ovarian steroid production at different stages of development, and possesses the capacity to secrete progesterone, androstenedione, testosterone, oestrone and oestradiol-17 β (dependent on species).

In vitro studies with isolated ovarian cell types have led to the development of the 'two cell hypothesis' to explain the cellular basis of oestrogen biosynthesis by the ovarian follicle (Falck, 1959; Channing and Kammerman, 1973; Baird, 1977; Armstrong, Leung and Dorrington, 1979; Leung and Armstrong, 1980; see Richards, 1980 for review). The two cell hypothesis states that although the theca can produce androgen from cholesterol (hamster: Makris and Ryan, 1977; rat: Fortune and Armstrong, 1977; human: Tsang, Young, Simpson and Armstrong, 1977; sheep: Moor, 1977), the granulosa cell cannot and requires androstenedione and/or testosterone from the theca in order to produce oestradiol (Dorrington *et al.*, 1975).

In support of the two cell theory, granulosa cell production of androgens has been shown to be negligible (rat: Fortune and Armstrong, 1977; pig: Evans, Dobias, King and Armstrong, 1981; horse: Channing, 1969b; human: Channing, 1969a; sheep: Moor *et al.*, 1975) and similarly, thecal production of oestrogen is considered to be low (rat: Fortune and Armstrong, 1978; pig: Tsang, Leung and Armstrong, 1979; cow: Lacroix, Eechaute and Leusen, 1974; human: Moor, 1977; Moor, Tsang, Simpson and Armstrong, 1979). Ovine thecal production of oestrogen was thought to be negligible (Moor, 1977), but other authors have postulated thecal cells may

contribute almost half of the total oestrogen production (Armstrong, Weiss, Selstam and Seamark, 1981). The possibility cannot be excluded that more reliable culture conditions for thecal preparations will resolve this inconsistency. Co-culture of both cell types, or of granulosa cells in the presence of androgen precursor, has shown a greater production of oestrogens than that secreted by the granulosa cells alone (human: Ryan, Petro and Kaiser, 1978; monkey: Channing, 1980; hamster: Makris and Ryan, 1975; horse: Ryan and Short, 1965; rat: Oakey and Stitch, 1967; cow: Lacroix *et al.*, 1974; sheep: Moor, 1977).

The corpus luteum can be regarded as the terminal stage of ovarian follicle development and is formed after ovulation by differentiation of granulosa and theca by a process known as 'luteinization' (Keyes, Gradsby, Yuh, and Bill, 1983). Dependent on species, the corpus luteum secretes oestrogens, progestins, inhibin and relaxin. Initially, inhibin was thought to be produced by the sheep corpus luteum (Tsonis *et al.*, 1988), but other authors disagree (Mann, McNeilly and Baird, 1989; Bramley *et al.*, 1992) and suggest production is by antral follicles (Campbell, McNeilly, Mann and Baird, 1991; Mann, McNeilly and Baird, 1992). The ovine corpus luteum therefore secretes mainly progestins with little or no androgen or oestrogens (Hansel *et al.*, 1973). The corpus luteum is under direct endocrine control and does not survive long in the absence of the pituitary hormones (Rothchild, 1981). Other luteotropic hormones of the corpus luteum, that is, those which promote the growth of the corpus luteum and stimulate secretion of progesterone (Astwood, 1941), include prolactin, oestrogen, prostaglandins and catecholamines (see Keyes and Wiltbank, 1988 for review).

1.5.3. Hormonal regulation

Steroidogenesis is controlled on at least two levels, the first concerns local (or intra) ovarian control through the action of steroidogenic products such as androgen, progesterone and oestradiol, which operate through ultra-short, or short-loop feedback mechanisms to regulate further steroid production. The second level of control of steroidogenesis is through hypophyseal regulation involving the action of gonadotrophins which operate through long-loop feedback mechanisms.

1.5.3.1. Ultra-short, or short-loop feedback control

In sheep, as a result of maturational changes, granulosa cells gain an increased capacity to produce and convert androgens to oestrogens (Baird and Fraser, 1974; McNatty, Baird, Bolton, Chambers, Corker and McLean, 1976; Armstrong *et al.*, 1981), such that the changing concentrations of plasma oestradiol provide an index of growth of large antral follicles (Moor *et al.*, 1975; McNatty, Gibb, Dobson, Thurley and Findlay, 1981b). The majority of oestradiol secretion comes from the ovary containing the oestrogen-active follicle, that is, the follicle capable of secreting oestradiol when isolated from the remainder of the ovary (Hay and Moor, 1975; England, Webb and Dahmer, 1981; Staigmillar, England, Webb, Short and Bellows, 1982). In the rat, however, thecal androgens are not only the precursors for oestradiol synthesis (Schomberg, Stouffer and Tyrey, 1976), but also stimulate further aromatase enzyme activity with high concentrations of androgen preventing LH inhibition of oestradiol secretion (Katz, Leung and Armstrong, 1979; Daniel and Armstrong, 1980; Hillier, Zeleznik, Knazek and Ross, 1980; Hillier, 1981). Androgens also stimulate granulosa cell progesterone synthesis (pigs: Haney and Schomberg, 1978; rats: Nimrod and Pearlsman, 1984; bovine: Shemesh and

Ailenberg, 1977) and stimulate pregnenolone secretion in bovine granulosa cells, the latter acting as a substrate for androstenedione production and thus oestradiol secretion (Fortune, 1986).

Progesterone is known to inhibit FSH-induced steroidogenesis *in vitro* in the rat (Schreiber, Nakamura and Erickson, 1980) and, unlike oestradiol (Adashi and Hsueh, 1982; Zhuang, Adashi and Hsueh, 1982), inhibits the induction of aromatase activity by FSH (Fortune and Vincent, 1983). Progesterone is also thought to have a suppressive effect on oestradiol in cows (Fogwell, Weems, Lewis, Butcher and Inskeep, 1978) and pigs (Chan and Tan, 1986). Interestingly, although the biosynthesis and secretion of steroid and peptide hormones involve different cellular mechanisms, factors such as IGF-1 and/or forskolin (an adenylate cyclase activator), which promote progesterone biosynthesis, also increase oxytocin production in granulosa cells from preovulatory follicles (cow: Meidan, Altstein and Girsh, 1992).

Oestradiol is a potent amplifier of FSH action, enhancing the ability of FSH to alter the number of its own binding sites (Richards, 1978) and thus potentiating its own production through a positive feedback loop mechanism. Oestradiol, through the interaction with androgen and gonadotrophins, regulates progesterone biosynthesis by inhibiting progesterone production and secretion in favour of further pregnenolone secretion, by inhibition of 3 β -hydroxysteroid dehydrogenase (pig: Thanki and Channing, 1978; cow: Haney and Schomberg, 1978; Williams and Marsh, 1978; Fortune and Hansel, 1979; rats: Leung and Armstrong, 1979; Magoffin and Erickson, 1981).

Recently, other intraovarian regulators, many found within the antral fluid of the follicle, have been shown to be important in the

regulation of ovarian steroidogenesis (see Ireland, 1987; Hammond, 1981 for review). Administration of steroid free ovine follicular fluid to ewes during the follicular phase of the oestrous cycle (Baird, Campbell and McNeilly, 1990), or to GnRH-treated anoestrous ewes (Hunter, Hindle, McLeod and McNeilly, 1988), results in the immediate inhibition of follicular development and ovarian secretion of oestradiol, inhibin, androstenedione and testosterone, providing possible evidence for the existence of other regulators of steroidogenesis. Candidates may include: FSH-binding inhibitor (Darga and Reichert, 1979), LH-binding inhibitor, which inhibits LH action (Sanzo and Reichert, 1982) and gonadotrophin surge inhibiting factor (GnSIF), an ovarian protein, found in follicular fluid, which is antagonistic to GnRH at the level of the pituitary gland (Sopelak and Hodgen, 1984; Fowler, Messinis and Templeton, 1990; Koppelaar, Tijssen and de Koning, 1992). In addition, follicular fluid contains other extra-ovarian protein hormones such as insulin (Rein and Schomberg, 1982), prolactin (Meloni, Ben-Rafael, Fatch and Flickinger, 1986) and prorenin (Gloriosco, Atlas, Laragh, Jewelwicz and Sealey, 1986). Many more factors of unknown molecular identities exist and of those possible intragonadal regulators which have been identified, their mechanisms of action and regulation are poorly understood.

Intraovarian growth factors, as well as possessing mitogenic effects within the ovary, are also known to affect steroidogenic function, either directly or in combination with other growth factor(s) and/or hormones. Perhaps the best understood growth factor is IGF-1 which can stimulate biosynthesis of progesterone and oxytocin by bovine granulosa cells (Schams *et al.*, 1988; Sauerwein, Mijamoto, Günther, Meyer and Schams, 1992) and enhance aromatase activity and thecal-interstitial androgen synthesis in rat granulosa cells (Adashi, Resnick, Svoboda and Van Wyk,

1985b; Hernandez, Resnick, Svoboda, Van wyk, Payne and Adashi, 1988; Cara and Rosenfeld, 1988). For these reasons, IGF-1 is thought of as an amplifier of gonadotrophin action (Adashi, Resnick, Svoboda and Van Wyk, 1986b). As well as being regulated by other growth factors (Mondschein *et al.*, 1988), IGF-1 action is also mediated by the gonadotrophins (Veldhuis and Demers, 1985; Veldhuis, Rodgers and Furlanetto, 1986; Hsu and Hammond, 1987). IGF-1 is known, either alone or in the presence of FSH, to increase inhibin production by rat granulosa cells (Zhang, Carson, Herston, Lee and Burger, 1987). IGF-1 production is thought to be partially mediated by oestradiol and the concentration of follicular fluid IGF-1, like oestradiol, shows some correlation to follicle size (Hammond, Baranao, Skaleris, Knight, Romanus and Rechler, 1985).

TGF- β is another growth factor which stimulates secretion of FSH from pituitary cells *in vitro*, enhances FSH-induced steroidogenesis in granulosa cells, and LH receptor formation under basal FSH conditions (Ying *et al.*, 1986b; Ying, Becker, Baird, Ling, Ueno, Esch and Guillemin, 1986a). Both TGF- β and TGF- α are important modulators of progesterone and oestradiol secretion (Adashi and Resnick, 1986; Dobson and Schomberg, 1987). In the cow, TGF- α has suppressive effects on theca cell progesterone and androstenedione production, whereas, in the absence of oestradiol, TGF β stimulates progesterone (Roberts and Skinner, 1991).

In contrast, FGF inhibits FSH-mediated induction of granulosa cell LH receptors (Mondschein and Schomberg, 1981) and FSH-induced aromatase activity (Baird, Esch, Mormede, Ueno, Ling, Bohlen, Ying, Wehrenberg and Guillemin, 1986). *In vitro*, FGF alone has no effect upon rat granulosa cell proliferation, but does act synergistically with insulin (Savion *et al.*, 1981). EGF inhibits the gonadotrophin stimulation of oestrogen production (Hsueh, Welsh and Jones, 1981) and also decreases LH receptor levels in

granulosa cells (Symanski and Schromberg, 1980). EGF involvement in regulating progesterone production remains controversial, with both stimulatory (Jones, Welsh and Hsueh, 1982) and inhibitory (Knetch and Catt, 1983) effects reported. The effect of EGF in regulating progesterone production may in fact be critically dependent on the relative concentration of EGF and FSH. In hamsters, EGF, IGF-1 and FGF, do not affect the accumulation of either androgen or oestrogen in follicular fluid and only EGF increased progesterone accumulation in the follicular fluid at specific stages of follicular development (Roy and Greenwald, 1991a). The role of most of the intra-gonadal hormonal, non-hormonal and regulatory factors in steroidogenesis are unknown.

1.5.3.2. Long-loop feedback control

Gonadotrophins, after interaction with their receptor sites, mediate their actions through a second messenger system including adenylate cyclase and cyclic adenosine monophosphate (cAMP; rat: Norderstrom, Rosberg and Roos, 1985; mouse: Carroll, Whittingham and Wood, 1991a; sheep: Weiss, Seamark, McIntosh and Moor, 1976; Adashi, Resnick and Jastorff, 1990). The response of ovarian follicular cells to gonadotrophins is dependent not only on the content of the gonadotrophin receptors, but also on the composition of the adenylate cyclase system, such that an increase in response to FSH, in terms of cAMP or adenylate cyclase production, can occur in granulosa cells without any change in the number of FSH receptors (Richards and Hedin, 1988).

The best evidence of gonadotrophin involvement in the control of steroidogenesis is the presence of gonadotrophin-specific receptors on various cell types within the follicle (see Hammerstein, 1974; Hsueh and Lapolt, 1992 for review). Both FSH and LH receptor numbers vary between

the pregnant and the non-pregnant animal (see Richards, 1978 for review). In preantral follicles, LH binds to theca whilst FSH binds to granulosa, allowing follicles to respond to both FSH and LH at this early stage of development (rat: Rajaniemi and Vanha-Perttula, 1972; Lee and Ryan, 1973; Richards, Ireland, Rao, Berneath, Midgley and Reichert, 1976; see Richards, 1980 for review; cow: Merz, Hauser and England, 1981; pig: Zeicik, Shaw and Flint, 1980; sheep: Carson *et al.*, 1979; Erickson, Magoffin, Dyer and Hofeditz, 1985). In the rat, oestradiol produced by granulosa cells then acts within the follicle stimulating granulosa cell proliferation (Goldenberg, Vaitukaitis and Ross, 1972), enhancement of FSH binding to granulosa cells (Richards, 1975; Richards and Midgley, 1976), further FSH-induced oestradiol secretion and induction of more theca LH receptors. The major effect of LH on thecal cells is to stimulate synthesis of androgens (rat: Armstrong and Papkoff, 1976; cow: Henderson, Kieboom, McNatty, Lun and Heath, 1984b). LH has only limited binding to granulosa cells of small antral and preantral follicles (Ryle, 1972; Midgley, 1973), but can bind to granulosa cells of large preovulatory follicles (pig: Channing and Kammerman, 1973; Channing and Kammerman, 1974; rat: Nimrod, Bedrak and Lamprecht, 1977; sheep: Webb and England, 1982b). In contrast, LH binding to preovulatory follicles causes a decrease in responsiveness to LH and FSH, although the mechanism is not fully understood.

FSH receptors are present and responsive at an earlier stage of granulosa cell differentiation, than are LH receptors (Armstrong *et al.*, 1981), since granulosa cells in preantral follicles possess FSH receptors, but do not gain LH receptors until antrum formation (mice: Eshkol and Lunenfeld, 1972; pig: Channing and Kammerman, 1974; sheep: Carson *et al.*, 1979). Receptors for FSH have been located exclusively on granulosa cells in many species (pig: Kammerman, Canfield, Kolera and Channing,

1972; cow: Merz *et al.*, 1981; rat: Leung and Armstrong, 1980; Richards and Rolfes, 1980; sheep: Weiss, Armstrong, McIntosh and Seamark, 1978; Carson *et al.*, 1979). FSH stimulates oestradiol production by granulosa cells by enhancing cholesterol availability and utilization in thecal cells, in common with LH (rat: Armstrong and Papkoff, 1976; sheep: McNatty *et al.*, 1975; see Ireland, 1987 for review). FSH induces the appearance of both its own receptor and the LH receptor on granulosa cells (Zelevnik, Midgley and Reichert, 1974), as well as being required for the maintenance of gonadotrophic receptors throughout follicle development (Richards, 1980). The ability of FSH to induce LH receptors on granulosa cells may occur through local growth factors, for example TGF- β enhances the effect of FSH in rats (Knetch and Feng, 1986).

FSH binding characteristics to granulosa cells do not differ between Booroola genotypes (McNatty, Lun, Heath, Hudson, O'Keeffe and Henderson, 1989b) and cAMP generation is consequently similar (Henderson *et al.*, 1985; McNatty, Lun, Hudson and Forbes, 1990b). However, ewes possessing the Booroola fecundity gene have follicles which possess LH receptors at an earlier size compared to follicles from non-carriers of the gene (Henderson, Kieboom, McNatty, Lun and Heath, 1985; Henderson *et al.*, 1986). This genotypic difference in LH receptor characteristics may influence the steroidogenic capacity of follicles and may be as a function of the difference in follicular development. Finnish Landrace ewes, like Booroola carriers, have significantly fewer granulosa cells per oestrogen-active follicle (Baird *et al.*, 1982; Scaramuzzi and Radford, 1983; McNatty *et al.*, 1985a; Driancourt and Jegou, 1985; Driancourt *et al.*, 1986; Webb *et al.*, 1989). In contrast, oestrogen-active follicles from Romanov ewes are different (Driancourt and Fry, 1988). Despite a lower number of granulosa cells per follicle, Finnish Landrace ewes (compared to Merino x Scottish

Blackface ewes) on a per cell basis produce more oestradiol *in vitro* (Webb *et al.*, 1989). Fec^B carriers, however, produce less oestradiol on a per follicle basis, although the total amount of oestradiol in the peripheral circulation does not differ between the Booroola genotypes (McNatty *et al.*, 1985a; McNatty, Kieboom, Mcdiarmid, Heath and Lun, 1986a).

1.6. Ovulation rate

Ovulation rate can be defined in two ways, either as the average number of oocytes released in animals that ovulate, or as the average number of oocytes released per oestrous cycle by a group of animals. The first definition is normally used as it excludes animals which fail to ovulate (Scaramuzzi and Radford, 1983).

1.6.1. Genetic and environmental control

The number of oocytes shed at ovulation varies widely both within and between breeds, and poses an upper limit to litter size in polytocous species. This variation in the number of ovulations is due to genetic and enviromental factors and the interaction between them (Land, 1977; Gunn and Doney, 1979; see Scaramuzzi and Radford, 1983; Cahill and Mauléon, 1980; Haresign, 1992 for review).

The enviromental factors known to affect ovulation rate in the ewe include nutrition (Knight, Oldham and Lindsay, 1975; see Downing and Scaramuzzi, 1991 for review), liveweight (Morley, White, Kenney and Davis, 1978); time of year (McKenzie and Terrill, 1937), social factors, for example introduction of the ram (Martin and Scaramuzzi, 1973), age and parity (McKenzie and Terrill, 1937).

Ovulation rate is known to respond to genetic selection, probably in an additive way (Hanrahan, 1980; Hanrahan and Piper, 1982, Hanrahan,

1987) and has a higher repeatability than either fertility (the number of ewes conceiving) or fecundity (the number of offspring per pregnancy) (Bindon and Piper, 1976). Ovulation rate poses a limit to the potential success of within breed selection (Bindon and Piper, 1976; see Hanrahan, 1992 for review), but since it is a trait of high heritability, cross-breed selection can be achieved (Hanrahan, 1986; Hanrahan *et al.*, 1987). In ewes, the increase in ovulation rate that occurs through either within breed, or by cross-breed selection is mostly accompanied by increased variability in the number of ovulations (Lahlou-Kassi and Mariana, 1984; Bindon and Piper, 1982). Breeds which exhibit high ovulation rates are called 'prolific' or 'high-fecundity' breeds and are a valuable genetic resource to study the physiological factors regulating ovulation rate in the ewe. The use of experimental animals of known genetic backgrounds maintained under defined and controlled environmental conditions is essential to the study of ovulation rate (Scaramuzzi and Radford, 1983).

1.6.2. The role of FSH

The role of FSH in the regulation of follicular growth and steroidogenesis has been discussed in previous sections, but emphasis will now be placed on the role of this gonadotrophin in governing the total number of follicles which ovulate in the sheep.

The possibility that differences in FSH may be causally related to ovulation rate has been investigated within and between breeds. For some highly prolific sheep breeds FSH is higher than in breeds of lower prolificacy (Cahill, 1981; Lahlou-Kassi and Mariana, 1984). Other workers find no such correlation between FSH and prolificacy in ewes (Bindon, Blanc, Pelletier, Terqui and Thimonier, 1979; Adams, Quirke, Hanrahan,

Adams and Watson, 1988; Driancourt, Philipon, Locatelli, Jacques and Webb, 1988; Driancourt and Fry, 1990).

The evidence for elevated plasma FSH concentrations in prolific sheep breeds therefore remains controversial (see Webb *et al.*, 1991 for review). In the Romanov breed and breeds of lower prolificacy, periovulatory FSH concentrations, when compared over specific time periods (prostaglandin induction of luteolysis to the onset of oestrus; during oestrus; from the end of oestrus for 24 hours) showed no difference between genotypes (Bindon *et al.*, 1979). A more detailed study by Cahill, Saumande, Ravault, Blanc, Thimonier, Mariana and Mauléon (1981), showed a greater FSH peak on day 2 of the cycle at a time when secretion of ovarian steroids and hence negative feedback is minimal. Similarly, sheep carrying the Booroola fecundity gene are thought to have a higher second FSH peak than control ewes (Bindon *et al.*, 1984). Significant differences in plasma FSH (during: the periovulatory period, the period coinciding with the LH surge and on day 2) have been reported for the prolific D'Man breed from Morocco (Lahlou-Kassi and Mariana, 1984). However Webb and England (1982a), found no significant differences in periovulatory plasma FSH between Finnish Landrace and Suffolk breeds.

The difficulty in interpreting some studies which compare FSH and ovulation rate have been confounded by using between breed comparisons or using animals from a different strain when within breed comparisons are made. Further complications in correlating physiological variables, such as peripheral gonadotrophin concentrations, involve the genetic variation that exists due to chance variation within individuals and variation within families, the so-called 'sire effect' (see Webb *et al.*, 1991 for review). Segregation of a population on the basis of their ovulation rate can cause problems in interpretation since genetic and environmental

variation can occur which is independent of the gene of interest (Haley, 1990).

1.6.3. Manipulation of ovulation rate

An alternative method of evaluating whether gonadotrophin secretion is an important determinant of ovulation rate has been to use techniques designed to manipulate the gonadotrophin status and then to investigate the effects of such treatment on ovulation rate (Haresign, 1985). Ovulation rate can be altered by using two different techniques, either by immunization (passive or active) against ovarian steroids and/or peptides, or by exogenous hormone treatment.

Passive immunization against oestradiol-17 β during the last 5 days of the oestrous cycle, can cause a doubling of ovulation rate (Scaramuzzi and Radford, 1983; Webb and Gauld, 1985b; Webb and Gauld, 1987). Active immunization against androstenedione (Scaramuzzi and Baird, 1977; Scaramuzzi, Davidson and Van Look, 1977), testosterone, oestrogens (Scaramuzzi, Baird, Clarke, Martensz and Van Look, 1980; Scaramuzzi *et al.*, 1981; Pathiraja, Carr, Fordyce, Forster, Land and Morris, 1984) and progesterone (Hoskinson, Scaramuzzi, Downing, Hinks and Turnbull, 1982) have all been reported to increase ovulation rate in sheep, although inconsistent effects are seen in cattle (see Bindon, Piper, Cahill, Driancourt and O'Shea, 1986 for review; Webb and Morris, 1988). The mechanism of action through which immunization against the ovarian steroids and/or peptides in sheep and cattle increases ovulation rate has yet to be fully elucidated. LH pulse frequency increases in ewes immunized against androgens and oestrogens, however, the pattern for FSH is more variable depending on the particular steroid that is being targeted (Martensz, Scaramuzzi and Van Look, 1979). FSH concentrations decreased in

androgen-immunized ewes, increased in oestrogen-immunized ewes, or remained unchanged in testosterone-immunized ewes, yet all are equally effective in raising ovulation rate (Webb and Gauld, 1985a). Recently, Campbell, Scaramuzzi, Evans and Downing (1991c) determined that FSH concentrations in androstenedione-immunized ewes did not alter during the oestrous cycle, and suggested that the discrepancy with other workers may be due to differences in immunogen, level of antibody or breed.

Both passive and active immunization against inhibin have increased ovulation rates (sheep: O'Shea, Cummins, Bindon and Findlay, 1982; Henderson, Franchimont, Lecomte-Yerna, Hudson and Ball, 1984a; Henderson, Al-Obaidi, Bindon, Hillard, O'Shea and Piper, 1986; Cummins, O'Shea, Al-Obaidi, Bindon and Findlay, 1986; Mann *et al.*, 1989; Al-Obaidi *et al.*, 1987; Cummins *et al.*, 1987; O'Shea, Andrews, Bindon, Hillard, Miyamoto and Sinosich, 1991; Wrathall, McLeod, Glencross and Knight, 1992; cattle: Price, Morris, O'Shea and Webb, 1987). In sheep, the increase in ovulation rate, through either passive or active immunization against inhibin, may be partially mediated by the effects on plasma FSH concentrations (McNeilly, Crow and Campbell, 1991), although direct action from the ovary cannot be excluded (Schanbacher, Schemm and Rhind, 1991). Exogenous inhibin is known to decrease ovulation rate through its depressive effect on FSH, but not LH (Findlay, Cummins, O'Shea and Bindon, 1981).

The second approach to manipulating ovulation rate in ewes has been through the use of exogenous substances, such as steroid enzyme inhibitors and releasing factors, although mainly through the use of gonadotrophins. A novel approach to increasing ovulation rate has been achieved by treatment using a steroid enzyme inhibitor (Epostane) in ewes (Webb, 1987; Webb, Baxter, McBride and McNeilly, 1992) and rats (Snyder, Beecham and Schane, 1984). Interestingly the effect in ewes was not



through an increase in peripheral FSH concentration (Webb *et al.*, 1992a). Work by McLeod, Haresign and Lamming (1982); McLeod, Haresign and Lamming (1983) using multiple injection or infusions of GnRH, lead to the suggestion that raising the concentrations of peripheral gonadotrophin may also alter the number of ovulations.

Infusion, or injection, of ovine FSH can increase the number of ovulations (sheep: Baird and Fraser, 1974; Jabbour and Evans, 1991; cattle: Wright, Geytenbeek, Clarke and Findlay, 1981; Chupin, Combarnous and Procureur, 1984). Human chorionic gonadotrophin (hCG), or LH itself, have also been used to increase ovulation rates (sheep: Braden, Lammond and Radford, 1960; Radford, Avenell and Szell, 1984; Bodin and Driancourt, 1990; Wierzchos, Tischner and Maffii, 1992; pigs: Dzuik and Baker, 1962; Dzuik, Hinds, Marsfield and Focker, 1964; cattle: Graves, 1968; Rajamahendran and Sianangami, 1992). FSH and hCG are known to act synergistically to enhance follicular and uterine development (Simpson, Li and Evans, 1951). The administration of FSH and LH to increase ovulation rate is thought to be dependent on the proportion, or ratio, of FSH to LH (Cognié, Chupin and Saumande, 1986; Wright, Bondioli and Grammer, Kuzan and Menino, 1991) and may explain the variability in ovulation rate response when LH has been injected during the follicular phase of the cycle, or during an induced follicular phase in anoestrous ewes (McNatty *et al.*, 1981a; McLeod *et al.*, 1983; Scaramuzzi and Radford, 1983). In sheep, the correlation between hCG-induced ovulation rate and endogenous ovulation rate is positive in non-prolific breeds, but not significant in prolific breeds (Driancourt and Fry, 1990).

Pregnant mare serum gonadotrophin (PMSG) is rich in FSH activity and can increase the ovulatory response in ewes (Robinson, 1951; Moore and Rowson, 1960; see Hunter, 1980 for review). Increasing doses of PMSG,

alone, or in combination with FSH, cause a superovulatory effect, but produces large variation in response (Gordon, Williams and Edwards, 1962; Ryan, Bilton, Hunton and Maxwell, 1984) and can actually decrease the ovulation rate due to the persistent effect of PMSG and the prolonged presence of large follicles (Shelton and Moore, 1962).

Interestingly, prolific sheep breeds show a greater ovulatory response to PMSG than non-prolific breeds (Smith, 1976; Piper and Bindon, 1979; see Bindon *et al.*, 1986 for review). Even within a breed, such as in the Booroola Merino, PMSG has a differential effect on ovulation rate, with homozygous carriers of the fecundity gene producing a greater response than non-carriers (Piper, Bindon, Curtis, Cheers and Nethery, 1982). In cattle, PMSG stimulates the formation of not only non-atretic follicles >3mm in diameter, but also of atretic follicles >6mm in diameter (Van den Hurk, Spek, Dijkstra, Van Vorstenbosch, Hulshof and Dieleman, 1992). The superovulatory response of cattle to PMSG can be enhanced by approximately 50% as a result of pretreatment with bovine somatotropin (BST; Gong, Bramley, Wilmut and Webb, 1993). In sheep, PMSG is thought to achieve the high ovulation rates by increasing the rate of growth of small recruited follicles and sustaining the growth of the larger follicles (Driancourt and Fry, 1992). In cattle, PMSG in combination with hCG, increases the ovulation rate and the number of viable superovulated oocytes obtained, but marked variation exists both between, and within, breeds (Scanlon, Screenan and Gorgon, 1968; Mauléon, Rey, Mariana and Benoit, 1970).

1.7. Summary

The number of lambs born per ewe termed 'litter size', has a marked effect on the biological and economic efficiency of sheep production

systems (Haresign, 1985). The upper limit to litter size in polytocous species is ovulation rate, which is dependent upon the number of follicles that develop and eventually ovulate. In sheep, follicular growth and development is a highly complex and coordinated event, regulated both by neural signals from the brain and by the interaction of hormones and/or growth factors from the hypothalamo-pituitary-ovarian axis. Marked genetic differences in prolificacy provide a powerful tool to help determine the control mechanisms involved in ovulation rate (Webb *et. al.*, 1991). Previous investigations into the mechanisms controlling ovulation rate using ewes possessing the Booroola fecundity gene have also attempted to establish the primary site of action of the gene. It is at present unresolved whether the primary site of action of the Booroola fecundity gene is exerted at the level of the pituitary gland or at the ovarian level. Further complications in determining the predominant site of action of the Booroola fecundity gene have arisen from studies which used animals that were of an unknown genetic background and, in some cases, compared different strains of animals.

The work in this thesis uses Booroola Scottish Blackface from discrete breeding lines, whose genetic relationship to other members of the group was carefully regulated. The aims of this thesis were to investigate possible factors, namely FSH, IGF-1 and inhibin, which may affect follicular growth and ovulation rate in the prolific Booroola Scottish Blackface ewe, in order to determine the site of action of the major gene known to be responsible for the genotypic differences in ovulation rate.

CHAPTER 2

Materials and Methods

2.1. Animals

2.1.1. Genetic basis of the Booroola

2.1.1.1. Origin of the Booroola

The Booroola Merino sheep was initially developed from the Non-Peppin strain of the Australian Merino by the Seeers Brothers of 'Booroola' Cooma, New South Wales, Australia. In 1958, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) continued this selection work on the Booroola Merino by selectively breeding ewes and annually purchasing sires from stud flocks whose matings proved to have no previous history of multiple births, although the duration and precise nature of the selection process is unknown (Newton-Turner, 1978). The performance of the Booroola ewe is now thought to arise from the action of a single, or major, gene called the 'Booroola fecundity gene' or 'Fec^B gene'. which increases ovulation rate, (Bindon, Piper and Evans, 1982; see Montgomery, McNatty and Davis, 1992a for review).

2.1.1.2. Booroola Scottish Blackface experimental lines

In order to investigate the mechanisms controlling ovulation rate, one of the major determinants of fecundity in sheep, the Fec^B gene was introduced into the native Scottish Blackface sheep by the Roslin Institute, Edinburgh. The experimental line breeding programme began in 1984 with the importation from Australia of several Booroola Merino rams which were homozygous carriers of the Fec^B gene. The

Booroola Merino rams were then crossed with Scottish Blackface ewes to produce an F_1 population. The F_1 population were crossed *inter se* to produce an F_2 generation, which was represented by either homozygous carriers, $Fec^B Fec^B$, or homozygous non-carriers, $Fec^+ Fec^+$, of the fecundity gene. The F_2 population were the foundation stock of the two lines, both lines being 50% Booroola Merino, 50% Scottish Blackface in their background genotype. The F_2 lines differed only at the Booroola locus and a short stretch of chromosome thus avoiding the spurious effects (see section 1.6.2.) attributed to the Booroola fecundity gene (Haley, 1990).

The mean genotypic ovulation rates are modified from the original segregation criteria for Booroola Merino genotypes as designated by Davis, Montgomery, Allison and Kelly (1982), such that homozygous carriers had ovulation rates > 5 , heterozygotes had ovulation rates of 3 or 4 and non-carriers had ovulation rates of 1 or 2 (Lee, personal communication). Booroola Scottish Blackface females have two ovulation rates recorded each breeding season after puberty, whilst males are genotyped by progeny testing after backcrossing to Scottish Blackface ewes. Female offspring from progeny testing have 75% Scottish Blackface and 25% Booroola Merino background genotype, with their putative Booroola genotype assigned by two measures of ovulation rate in their second season. In order to identify their genotype, males were crossed with the native Scottish Blackface ewes and the ovulation rate of the progeny monitored twice over three seasons.

A traditional 'backcross' would involve males from the F_1 population (all heterozygotes) being crossed back to Scottish Blackface ewes. Approximately half of the progeny from this test would be the

heterozygotes and the remainder, non-carriers of the Booroola fecundity gene. The F₁ backcross population would be 25% Booroola and 75% Scottish Blackface. In the breeding programme identification of the F₂ male heterozygotes was achieved by crossing to the native Scottish Blackface ewe to produce progeny which were 25% Booroola and 75% Scottish Blackface. It is the progeny produced from these F₂ males which were used in some of the studies described in this thesis and these animals are referred to as 'backcross' animals. The animals were maintained at the Roslin Institute (Edinburgh) farms (Dryden or Blythbank) in southern Scotland, and managed on traditional, upland, grassland sheep farming management.

The highly prolific Booroola Merino serves as a genetic resource for both industry and research into ovine genetics and physiology. The Booroola Scottish Blackface flock serves as a research model to understand the genetic and physiological control of follicular growth and ovulation rate rather than to investigate the practical applications of the gene (Haley, 1990).

All experimental procedures conducted for this thesis are in compliance with the Animals (Scientific Procedures) Act, 1986.

2.2. Surgical Procedures

2.2.1. Blood sampling

Ovine blood samples were obtained either by jugular venepuncture, using vacutainer or syringe, or by cannulation of the ovarian vein. Ovarian venous blood was collected in a sterile graduated tube for 10 minutes. The ovarian vein blood flow per minute was corrected for haematocrit (Collett, Land and Baird, 1973). The blood was

then allowed to clot overnight at room temperature and the serum collected by decantation after centrifugation for 25 minutes.

2.2.2. *Laparoscopy*

The method of laparoscopy performed in these studies was modified from Holland, Bindon, Piper, Thimonier, Cornish and Radford (1981). All the animals were fasted from food and water for 24 hours before anaesthesia was induced by intra-venous injection of a 10% aqueous solution of sodium thiopentone (1g Intraval Sodium/ 90kg liveweight; R.M.B. Animal Health Ltd, Dagenham, Essex) and maintained by 2% Fluorthane (Coopers Animal Health, Crewe, Cheshire) in air passed through an endotracheal tube. A long-acting antibiotic (Duphaphen L.A., Penicillin; Dubhar Veterinary Ltd., Hedgeend, Southampton) was administered intra-muscularly to the animal which was then placed on the surgical table and maintained in a recumbent position with the hindquarters higher than the forequarters. Surgical preparation of an area of skin, approximately 10-15cm anterior to the mammary gland, enabled two short (1.5cm) incisions to be made either side of the midline, approximately 20cm apart.

The laparoscope trochar and cannula were inserted through one of the incisions and into the peritoneal cavity. The probe trocar and cannula were inserted into the remaining incision. The abdominal cavity was inflated by using medical grade nitrous oxide to enable observation of the ovaries. After all observations were recorded, the excess peritoneal gas was expelled before Michel clips were used to close the incisions, and a topical antibiotic spray applied to the general area. The whole procedure took approximately 25 minutes and had no long term adverse effect on the animals.

2.2.3. Ovariectomy

The animals which underwent ovariectomy were fasted, surgically prepared and anaesthetized as described in section 2.2.2. Once the animal was in a recumbent position on the surgical table, a mid-ventral incision was made, approximately 10cm in length. The ovaries were located and placed outside the body cavity to enable ligation of the blood vessels that supply it. Each ovary was in turn removed with a scalpel and the wound closed using degradable sutures (Dexon Plus Davisgeck, Cyanamid, Hampshire), before the area was sprayed with a topical antibiotic spray.

2.3. Radioimmunoassays

2.3.1. Assay buffers

Assay buffers were prepared using radioimmunoassay (RIA) grade chemicals obtained from Sigma Chemical Company, Poole, Dorset. The type of buffer which was used in a radioimmunoassay was dependent on the active principle being measured. The gonadotrophin radioimmunoassays utilized bovine serum albumin-general assay diluent buffer (BSA-GAD), whilst steroid RIAs required a phosphate-gelatin buffer (phosgel) and those that were used to measure the peptide, insulin-like growth factor-1 required a BSA-GAD sodium dihydrogen orthophosphate-based buffer. Both the BSA-GAD and phosgel buffers are derivations of a 0.5M phosphate base which was prepared by dissolving 716g disodium hydrogen orthophosphate into 4 litres of purified water obtained from a Waters Milli-Q purification system (Millipore Corporation, Milford, MA). Sodium dihydrogen orthophosphate (78g dissolved into 1 litre purified water) was added to

the disodium hydrogen salt solution until a pH of 7.5 was obtained using a previously calibrated pH meter. The disodium hydrogen salt solution at pH 7.5 is commonly referred to as the 'stock phosphate buffer solution'.

The 0.1% BSA-GAD buffer used for gonadotrophin assays contained 200ml of stock phosphate buffer solution, 1.8 litre of water into which was dissolved 18g sodium chloride and 2g of bovine serum albumin (fraction V; Sigma). Bacterial contamination was prevented by the addition of 0.01% thimerosal before the solution was stored at 4°C. The phosgel buffer (0.1%) was prepared similar to that stated above, but the bovine serum albumin was replaced by 2g of swine skin gelatin (300 bloom). The swine skin gelatin was dissolved in 400ml of the buffer at 50°C to aid it dissolving, before it was added to the rest of the buffer. The IGF-1 assay buffer was prepared by adding 4.14g of sodium dihydrogen orthophosphate, 3.72g diaminoethanetetra-acetic acid disodium salt (EDTA; Fisons, Loughborough), 10ml of 2% sodium azide, 500µl of Tween 20 and 1g BSA-GAD to approximately 800ml distilled water and 2M sodium hydroxide added until the solution was at pH 7.5, before additional distilled water was added to result in a 1 litre solution.

2.3.2. FSH

FSH was measured in a homologous double antibody radioimmunoassay system (McNeilly, Fordyce, Land, Martin, Springbett and Webb, 1988) supplied by National Institute of Arthritis, Metabolism and Digestive Diseases, NIAMDD. This assay is routinely used at the Roslin Institute as follows. NIAMDD-oFSH-RP1 stock (10ng/ml) was diluted in 12 X 75 polystyrene tubes (LIP, Shipley) to a final volume of 500µl, using 0.1% BSA-GAD assay buffer, to give a range of standard

concentrations (0.05 to 4ng/tube). Unknown samples were assayed at a known volume and diluted up to 500 μ l using assay buffer. Specific antisera (R-anti-oFSH-I-1), which was stored at a concentration of 1:1,000 at -40°C was diluted to 1:20,000 in 0.1% BSA-GAD and added at 200 μ l per tube (except to tubes which would only receive radiolabelled FSH, that is, 'totals', or to tubes in which first antibody would not be added in order to obtain a measure of non-specific binding). Ovine FSH was iodinated by the lactoperoxidase method of Thorrel and Johansson (1971) and the radiolabelled FSH (NIAMMD-oFSH-I-1) at a dilution of 20,000cpm, which had been iodinated with iodine-125 (¹²⁵I), was added to the assay at 100 μ l per tube. All the tubes were then vortexed and incubated overnight at 27°C.

The antibody-bound and unbound FSH were separated by carrier serum in the form of NRS, or Normal Rabbit Serum (Scottish Antibody Production Unit, SAPU, Law Hospital, Carluke, Lanarkshire, Scotland) and by use of a second antibody, Donkey Anti-Rabbit Serum (DARS; SAPU). NRS was added (100 μ l) to all tubes (except totals) at an initial dilution of 1:300 in 0.1% BSA-GAD. DARS was added (200 μ l to each tube except totals) at an initial dilution of 1:45 in 0.1% BSA-GAD plus 10% 0.1M EDTA. The tubes were then incubated for 24 hours at 4°C, before the addition of 1ml of 0.1% BSA-GAD and centrifugation at 2,000g for 30 minutes at 4°C. The supernatant was discarded and the tubes allowed to dry before the activity in the remaining pellet counted on the automatic gamma counter (LKB Ltd.).

2.3.3. *Oestradiol-17 β*

Oestradiol-17 β concentrations in sera required extraction by affinity chromatography (Webb, Baxter, McBride, Nordblom and Shaw,

1985). Serum samples were placed in screw capped glass tubes, together with 100 μ l of CNBr-activated Sepharose 4B (Pharmacia) covalently linked to oestradiol antiserum, 10 μ l of tritiated oestradiol (^3H -oestradiol-17 β) at 1,000 counts/100seconds (Amersham International plc.) and 10ml of purified water. The samples were mixed 'end over end' overnight at room temperature. The contents of each tube were poured on to prewashed chromatography soda glass columns (10 x 20mm) with glass sinter discs. The antibody-Sepharose residues were washed with 20ml of water and the eluates allowed to run to waste with the residual water being expelled under positive pressure. The extracted oestradiol was then eluted with 3mls of 90% methanol into 16 x 125mm glass tubes. The methanol was evaporated using a Buchler vortex evaporator (Galenkamp) at 40°C and the oestradiol extracts reconstituted in 1.8ml phosphate buffered saline containing 0.1% gelatin, by mixing at 40°C for 40-50 minutes. The extraction efficiencies were estimated by counting the ^3H activity in a 500 μ l aliquot of each reconstituted sample, which was placed in a plastic minivial together with 4ml of Scillant (Optiphase X; Fisons) and counted on a Rackbeta 1211 counter (LKB Ltd.) for 5 minutes. The counts were then compared to those obtained in samples where 10 μ l of ^3H -oestradiol-17 β was mixed with 500 μ l of phosgel buffer and 4ml of scintillant, minus background activity, which was estimated by counting vials containing only 500 μ l of phosgel buffer and scintillant.

Oestradiol-17 β concentration in ovine serum, follicular fluid and culture media samples were measured by radioimmunoassay, which is routinely used at the Roslin Institute (Webb *et al.*, 1985) and is described as follows. Oestradiol stock standard (Sigma), at a concentration of 100pg/ml was stored at -40°C. The oestradiol stock was rapidly thawed

and diluted into glass tubes to a final volume of 500 μ l, using phosgel buffer (0.1%), to give a range of standard concentrations (0.5 to 48pg/tube). Unknown samples, of known volume, were diluted up to 500 μ l using assay buffer. Specific antisera (R48; raised in rabbits against oestradiol-11 β -succinyl-bovine serum albumin), which was stored at a concentration 1:1,000, was diluted to 1:40,000 in phosgel buffer and added at 200 μ l per tube (except to tubes designated as totals and tubes used to measure non-specific binding). Radiolabelled oestradiol (125 I-17 β -oestradiol-11 α -tyrosinemethylester) at a dilution of 15,000cpm, which had been iodinated with iodine-125 by the method of Hunter, Nars and Rutherford (1975), was added to all tubes at 100 μ l per tube. All the tubes were then vortexed and incubated for at least 2 hours at room temperature.

The antibody-bound and unbound oestradiol were separated by the addition of 100 μ l per tube NRS at 1:400 dilution, in assay buffer, and 100 μ l per tube of DARS (1:40 initial dilution) plus 10% EDTA in assay buffer. The tubes were then incubated for 24 hours at 4 $^{\circ}$ C, before the addition of 1ml of 0.1% phosgel assay buffer and centrifugation at 3700g for 25 minutes at 4 $^{\circ}$ C. The supernatant was discarded and the tubes allowed to dry before the activity in the remaining pellet counted on the automatic gamma counter.

2.3.4. Progesterone

Progesterone was measured by radioimmunoassay according to the method of Corrie, Hunter and MacPherson (1981); Webb *et al.*, (1988) and Law (1991) and is routinely used at the Roslin Institute. The progesterone standard, kept in 5ng/ml aliquots at -40 $^{\circ}$ C (Sigma), was diluted in glass assay tubes to a final volume of 500 μ l using phosgel

assay buffer and 50 μ l of serum (from an ovariectomized ewe). The standard concentrations of progesterone used in this assay ranged from 7.8 to 1,000pg/tube. Unknown samples were assayed at a known volume and diluted to 500 μ l using the assay buffer. Specific antiserum (R31/8 rabbit antiprogestosterone), was diluted to 1:16,000 and added at 200 μ l per tube (except to tubes designated as totals or non-specific binding tubes). The radiolabelled progesterone (125 I-progesterone-11 α -glucuronide-tyramine), which had been iodinated with iodine-125 by the method of Corrie *et al.* (1981), was added to the assay (100 μ l per tube) at approximately 12,000cpm. All the tubes were then vortexed and incubated for 2 hours at room temperature.

The antibody-bound and unbound fractions of progesterone were separated by the addition of 100 μ l per tube of NRS at 1:300 dilution in assay buffer, and 100 μ l per tube of 10% EDTA in assay buffer plus DARS (1:35). The tubes were vortexed and incubated overnight at 4 $^{\circ}$ C, before the addition of 1ml of phosgel assay buffer to each tube and centrifugation at 3,700g for 25 minutes. The supernatant was discarded by decantation and the tubes allowed to dry before the activity in the remaining pellet counted on the automatic gamma counter (LKB Ltd.).

2.3.5. IGF-1

Concentrations of IGF-1 were measured by radioimmunoassay as described previously (Armstrong, Duclos and Goddard, 1990), with modification of the serum sample extraction procedure (Enright, Chapin, Moseley, Zinn, Kamdar, Krabill and Tucker, 1989), which uses a mixture of ethanol: acetone: acetic acid (60:30:10). The radioimmunoassay is routinely used at the Roslin Institute (Gong, Bramley and Webb, 1991) and was validated for sheep (see below). The

stock solution of IGF-1 (Beckham, Essex) was stored at -80°C in $100\mu\text{l}$ aliquots at a concentration of 10ng/ml . The IGF-1 stock was thawed and diluted into plastic tubes to a final volume of $200\mu\text{l}$ per tube, using assay buffer, to give a range of standard concentrations (200 to $2,000\text{pg/tube}$). Unknown samples were assayed at a known volume and diluted to $200\mu\text{l}$ using assay buffer. Specific antiserum (anti-human IGF-1 antiserum R2/2 raised in rabbits), which was stored at -80°C in 1ml aliquots, was diluted to 20mls in assay buffer to give a dilution of 1:10,000 and $200\mu\text{l}$ per tube added to all tubes except totals and tubes used to measure non-specific binding. All the tubes were vortexed and incubated overnight at 4°C . ^{125}I -IGF-1, $100\mu\text{l}$ per tube at 20-40,000cpm, was added to all tubes and incubated overnight at 4°C .

The antibody-bound and unbound fractions of IGF-1 were separated by the addition of $100\mu\text{l}$ per tube of NRS (1:200) and DARS (1:20) in assay buffer, which after mixing, was then left to incubate for 3 hours at room temperature. 1ml of polyethylene glycol (4%) was added to all tubes, except totals, before incubation at room temperature for 30 minutes. After incubation, the tubes were centrifuged for 15 minutes (2,800rpm at 4°C) before $100\mu\text{l}$ of starch (10%w/v) was added to each tube (except tubes designated as totals) and the tubes centrifuged for a further 15 minutes. The supernatants were aspirated and the pellet counted on the automatic gamma counter.

2.3.5.1. Validation

It is now known that dissociation and removal of insulin-like growth factor binding proteins (IGFBPs), from IGF-1 is essential before serum samples can be reliably assayed (Daughday, Kapadia and Mariz,

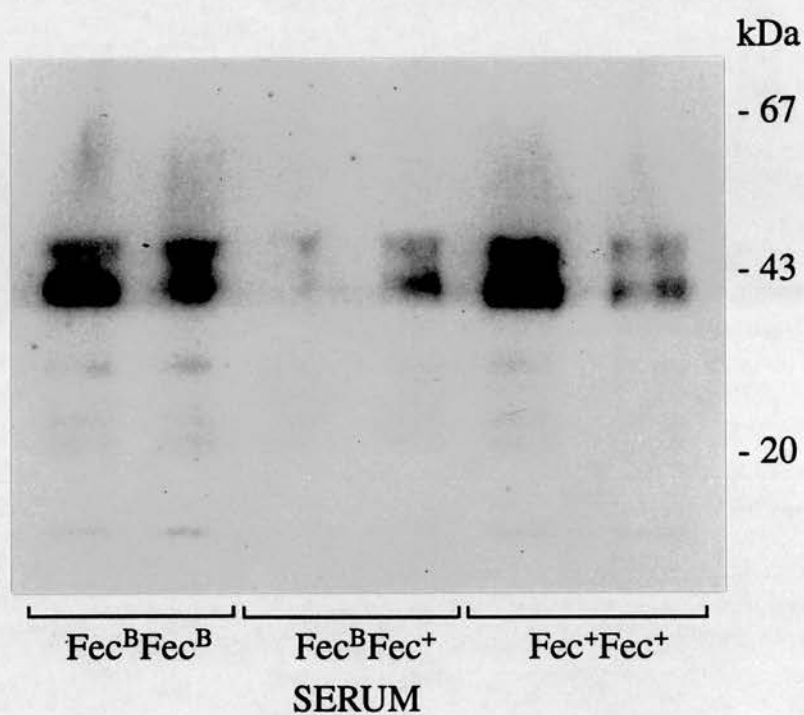
1987). Since a number of different methodologies have appeared in the literature, concentrations of IGF-1 were measured in the serum of two different species using a variety of IGFBP extraction techniques. These were: (a) acid-ethanol (Daughaday, Mariz and Blethen, 1980); (b) acid-ethanol cryoprecipitation (Breier, Gallaher and Gluckman, 1991); (c) ethanol-acetone-acetic acid (EAA; Enright *et al.*, 1989); (d) Sep-Pak (Goddard, Wilkie and Dunn, 1988); (e) acid-ethanol microseparation (Pell, personal communication). The results show wide variation in the concentration of IGF-1 after extraction of the IGF-BPs by these different methodologies. The presence of IGFBPs cause interference with the IGF-1 radioimmunoassay by competing with the antibody for the radioactive label such that there is an overestimation of the IGF-1 concentration. The results (Table 2.1.) show that EAA gives the lowest value for the IGF-1 concentration and suggests that the other methods are not as efficient at removing IGFBPs.

Since the ethanol-acetone-acetic acid method (Enright *et al.*, 1989) has been validated for bovine serum (Gong, 1992) and is routinely used in our laboratory, it was decided to extend the validation by checking the efficiency of this IGF-BP extraction procedure using radioligand blotting (Hossenlopp, Seurin, Segovia-Quinson, Hardoun and Binoux, 1986). Figure 2.1. shows that almost all of the BPs in ovine serum are removed by the EAA method. This suggested that the method would be appropriate for ovine serum and confirms the results shown in Table 2.1.

Therefore, using the EAA method, the dilution-response curve of extracted sera pooled from Booroola Scottish Blackface ewes (n=4 per genotype) were found to be parallel to the standard curve (see Figure 2.2.). In addition, the mean and standard error (\pm s.e.m.) percentage

Figure 2.1. Autoradiographs of western ligand blots probed with ^{125}I -IGF-1. Panel (A) shows duplicate samples of pooled ovine serum from the three genotypes of the Booroola fecundity gene after exposure for 10 days. (B) shows ovine serum extracted for IGF-1 using the EAA method to show the efficiency of the removal of IGFBPs. (a) and (b) show autogradiographs exposed for 5 and 28 days respectively. Molecular weight markers are in kiloDaltons (kDa) are shown.

A



B

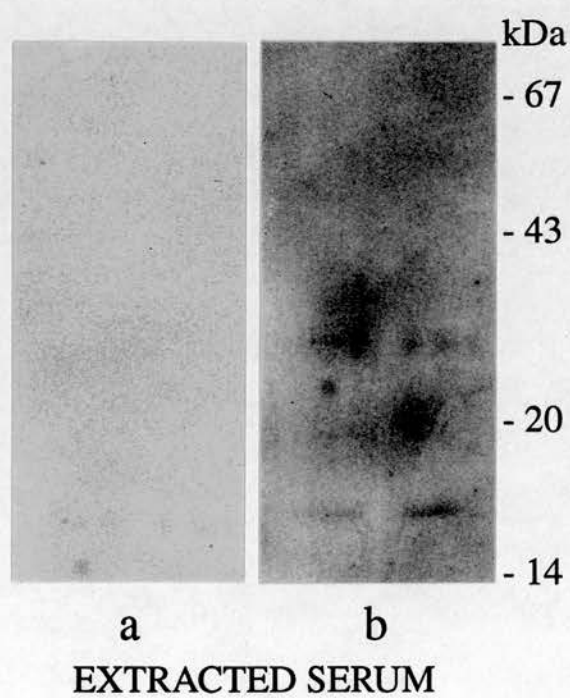


Figure 2.2. Comparison of a standard curve for insulin-like growth factor 1 (IGF-1) and dilution-response curves of extracted (diluted 1:10) from serum obtained from twelve ewes during the breeding season which were either homozygous carriers, heterozygous or non-carriers of the Booroola fecundity (Fec^B) gene (n=4 per genotype). For each sample, the amount of radioactive label bound to the antibody (B) is expressed as a percentage of the maximum amount of radioactive label (B_0) which can bind to the antibody.

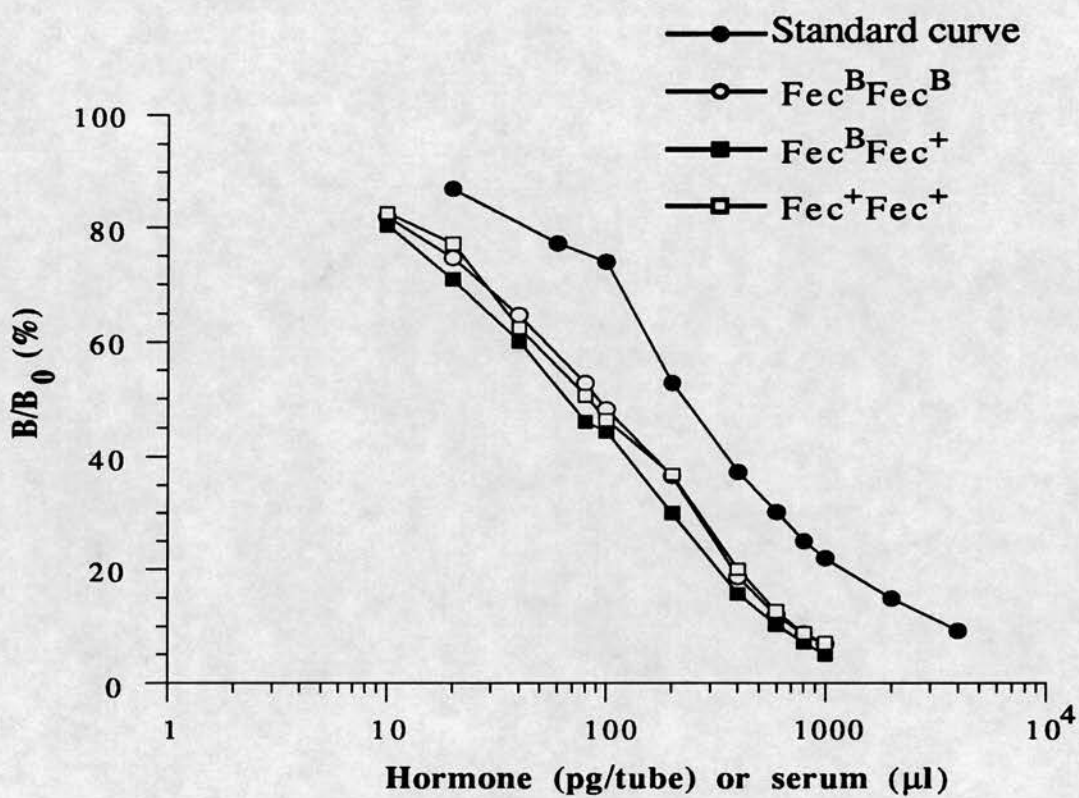


Table 2.1. Mean (\pm s.e.m.) peripheral concentrations of IGF-1 (ng/ml) in serum obtained from cattle and sheep after extraction using different procedures to remove IGF-BPs.

Method	Cattle	Sheep
acid-ethanol	336.7 \pm 20.3	146.3 \pm 1.5
acid-ethanol cryoprecipitation	312.3 \pm 11.3	128.0 \pm 8.2
ethanol-acetone-acetic acid	164.4 \pm 12.3	60.2 \pm 2.8
sep-pak	239.0 \pm 10.9	116.0 \pm 3.5
acid-ethanol microseparation	194.9 \pm 5.1	116.0 \pm 4.7

recovery of radioactive IGF-1, using 5, 10 and 20 μ l, was checked and found to be $104 \pm 3\%$.

2.3.6. *Statistics*

Radioimmunoassay is based on the competition between a fixed amount of labelled hormone and unlabelled hormone for a limited number of antibody binding sites. The number of counts obtained from the automatic gamma counter were analysed using the Assayzap programme on the Apple Macintosh which plots a curve for the standard samples and expresses the potencies of the unknown samples. In order to assess the variation both between (inter) and within (intra) assays, the coefficients of variation (CVs) are quoted. Apart from the variation that is incurred in the assay(s), it is usual practice to include a measure of the minimum amount that the assay can detect, that is, the sensitivity of the assay (see relevant Chapters). The sensitivity of the assay was calculated by the Assayzap programme (Taylor, Elsevier, Biosoft, USA) on the Apple Macintosh computer.

The inter-assay coefficient of variation (CV_{inter}) was calculated by using the estimates of the potency for the quality control samples in each assay by;

$$CV_{\text{inter}} = \frac{SD}{X} \times 100$$

where SD is the standard deviation of the potencies for the quality control samples and X is the mean of those potencies. The mean CV_{inter} for all the assays was calculated and expressed as a percentage.

The intra-assay coefficient of variation (CV_{intra}) was calculated from the potencies from 10 random samples in each assay using the formula;

$$CV_{\text{intra}} = \frac{100S}{X}$$

where X is the mean of all the samples used in the assay and,

$$S = \sqrt{\frac{\sum d^2}{2n}}$$

where d is the difference between the duplicates and n is the number of pairs of samples in the assay.

2.4. Follicular fluid

2.4.1. Collection

At ovariectomy (see section 2.2.3.), the ovaries were collected in a vessel containing approximately 10ml Leibovitz L-15 culture media (Gibco BRL, Paisley). The follicles were manually dissected before being placed in a 1.8ml Bio-freeze vial (Costar, Cambridge) and submerged into liquid nitrogen before being removed and stored at -80°C . Later, individual follicles were rapidly thawed and their diameters measured by using Vernier calipers. Each follicle was then burst into an individual well (Costar, Cambridge) containing 1ml of 0.3% BSA-GAD (the BSA-GAD solution was formulated as described in section 2.3.1., except that 6g of bovine serum albumin was used). The solution from each well was then placed into a 1.8ml plastic Eppendorff tube (Alpha

Laboratories, Hampshire) and centrifuged for 15 minutes at 4°C. The supernatant was collected and stored at -20°C for subsequent assays.

2.4.2. Calculation of hormone concentration

There is a linear relationship between follicle diameter and antral fluid volume (England *et al.*, 1981) such that;

$$y = 1.69x - 0.52$$

where y is the follicular diameter and x is the cube root transformation of the antral fluid volume. The dilution factor involved in bursting the follicle into 1ml can be calculated from the following equation;

$$\text{Dilution factor} = \frac{\text{antral fluid volume} + 1000}{\text{antral fluid volume}}$$

The concentration of hormone (ng/ml of sample) calculated from the radioimmunoassay is converted to concentration of hormone (ng/ml of follicular fluid) using the following equation;

$$\text{Hormone (ng/ml follicular fluid)} = \text{Hormone (ng/ml sample)} \times \text{Dilution factor}$$

2.5. In vitro whole follicle culture

The methodology for the culture of whole follicles was as described previously by Nayudu and Osborn (1992) after modification for the sheep. The ovaries were aseptically removed at ovariectomy (see section 2.2.3.) and placed in Leibovitz-L15 (Life Technologies) supplemented with 2mM glutamine (Sigma). All subsequent work was carried out in sterile conditions inside a M.D.H. class 2 biological safety cabinet (Crowthorne, Glasgow).

Each pair of ovaries was placed into a 60x15mm diameter plastic culture dish (Costar, Cambridge) containing Leibovitz-L15. The ovaries were manually dissected to remove the small follicles (<1mm in diameter) using two 28 gauge needles attached to 1ml syringes and by viewing under a light microscope (x40 magnification). After dissection, follicles were kept at 37°C in a humidified chamber with a continuous flow of 5% carbon dioxide in air and placed in 300µl alpha Minimal Essential Medium, (αMEM; Life Technologies), supplemented with 25mM sodium hydrogen carbonate (Sigma) and 5% lamb serum (initially purchased from Life Technologies, but later obtained from prepubertal lambs at Dryden, Scotland), which was then adjusted to 270mOsm using a calibrated osmometer. This intermediate step, between dissection and culture, allowed the follicles to be graded. The criteria used for acceptable quality were a spherical oocyte, distinct follicle wall and a distinct layer of thecal cells. The mean diameter of each isolated follicle was measured with an ocular micrometer, whose limit of detection of measurement was 10µm.

The follicle culture media was αMEM with 25mM sodium hydrogen carbonate, 5% lamb serum, 5µgml⁻¹ bovine insulin (Sigma), 10µgml⁻¹ human transferrin (Sigma), 2mM glutamine, NIAMDD-oFSH-14 (of varying doses depending on the experiment), and 1% sodium pyruvate (Sigma). The supplements were stored at -80°C and after their addition to the rest of the culture media, the media were sterilized by filtration using a 0.22µm Millex-GV filter (Millipore, Watford). A known volume of follicle culture media was added to a plastic Nucleon well (Life Technologies), containing a 12mm diameter Millicell-CM culture insert (Millipore), and was equilibrated inside a humidified chamber with a continuous flow of 5% carbon dioxide in air (BOC, Glasgow). The culture

inserts were recycled according to the method of Bell and Quinton (1990). The quality and diameter of the follicles was measured daily, although the effect of leaving the follicles undisturbed for several days was tested (see Chapter 5). Day 0 was designated the first day of culture and the culture media was changed on day 2 and either on day 3 or day 4 as stated. The culture, preincubation and dissection medias were stored at -20°C for subsequent assay.

In order to confirm that the techniques employed were performed in sterile conditions, agar plates, used routinely at the Roslin Institute, which had been designated into quarters and coated with either: double distilled deionised water (Millipore Corporation, Milford, MA); culture media; dissection media or holding media, were incubated for several days inside the incubator. No signs of infection were observed on the agar plates, but as a further precaution, control Nucleon wells, containing either holding media or culture media, were incubated alongside Nucleon wells containing cultured follicles. Follicles after either dissection or various lengths of time in culture, were prepared for histological examination as described below.

2.6. Histological preparation

The material was 'fixed' by placing them into either aqueous Bouins' fixative for a minimum of several hours, or into ethanol: acetic acid (3:1) for at least one hour followed by transfer to 4% paraformaldehyde for at least one hour. In both cases, the material was then placed into 70% ethanol for subsequent processing. When the material was removed from the 70% alcohol it was placed into 90% industrial methylated spirits (IMS; Charles Tennant, Glasgow) for 60 minutes which was replenished after 30 minutes. The material was

transferred to 95% IMS for 60 minutes (which was replenished after 30 minutes), then placed into absolute alcohol (Haymans Ltd, Essex) before being transferred into Toluene for 30 minutes. After being embedded into paraffin wax (Paraplast; Oxford Laboratories, Oxford), the material was placed into a heated vacuum oven and the wax changed three times every 30 minutes. The paraffin wax block containing the material was allowed to cool before being serially sectioned at 6 μ m, and the sections mounted on glass slides which had been pretreated with gelatin to aid adhesion.

The sections were stained with haematoxylin and eosin. Briefly, the sections were 'dewaxed' by placing them into xylene (BDH, Leicestershire) for 15 minutes, before being quickly immersed several times into absolute alcohol, 90% IMS, 70% IMS and water. The slides were placed into the haematoxylin stain (BDH, Leicestershire), left for 5 minutes, rinsed quickly in water then placed in acid alcohol before being left for 3 minutes in Scotts tap water solution (STWS). STWS is made from 1 litre of tap water, 20g of magnesium sulphate and 35g of sodium bicarbonate. The slides were then transferred to running water and kept immersed for three minutes before being placed into the eosin stain (G.T. Guer, London) for several minutes. The slides were then rinsed with water, before being immersed into potassium alum for 2 minutes before being washed thoroughly with water and 'rehydrated', ie. placed serially into 70% ethanol, 90% ethanol and absolute alcohol, before being immersed for 15 minutes into the xylene solution. D.P.X. mountant (BDH, Leicestershire) was added dropwise to each slide and a coverslip placed over the top. The slides were then allowed to dry before being wiped clean and viewed under the light microscope.

2.7. Statistical analyses

All normally distributed data, as assessed from the Statworks package on the Apple Macintosh, was expressed as arithmetic means with the corresponding s.e.m. Data which was not normally distributed, as assessed by the Statworks package on the Apple Macintosh, was transformed to natural logarithms to allow statistical analyses, before being represented as geometric means (\pm s.e.m.). For normally distributed data, differences between the means was tested by Student's t test or by analysis of variance allowing for multiple comparisons. Correlation coefficients (r) were calculated using the Minitab programme on the Apple Macintosh and the r value was compared using correlation coefficient statistical tables (Fisher and Yeates, 1963).

CHAPTER THREE

Relationship between FSH and ovulation rate during oestrus and seasonal anoestrus

3.1. Introduction

An hormonal basis for the increased ovulation rate in the Booroola Merino has not been fully established although the possibility that ovulation rate may be causally related to differences in concentrations of plasma gonadotrophin and/or hypothalamic/pituitary function has been investigated (see sections 1.4.2. and 1.7.2.). Observations on peripheral FSH being responsible for controlling ovulation rate are unclear. In a study around the time of luteolysis, Booroola Merino ewes ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$) had significantly higher mean peripheral FSH concentrations than controls ($\text{Fec}^{+}\text{Fec}^{+}$), while $\text{Fec}^{\text{B}}\text{Fec}^{+}$ ewes had intermediate FSH concentrations (McNatty *et al.*, 1987). In contrast, Bindon *et al.* (1985) found no significant difference between ewes with the Booroola gene compared to ewes lacking the gene between 96 and 24 hours before the preovulatory gonadotrophin surge. The small differences observed by McNatty *et al.* (1987) could be due to sire effects (see section 1.6.2.) which are not fully accounted for in the analyses or by the way that the females were selected. Conversely, the lack of differences observed by Bindon *et al.* (1985) could be due to comparison of Booroola Merinos and Merinos of a different strain.

Clearly the establishment of defined experimental lines (section 2.1.1.2.) at the Roslin Institute, Edinburgh, provide a unique opportunity to resolve discrepancies in the literature. The strictly controlled breeding programme has enabled animals to be chosen, whose genetic background is well documented and whose genetic relationship to other members within

the same experimental group can be carefully regulated. An additional advantage in using animals which have been carefully selected from discrete breeding lines is it provides a rare opportunity to study the physiology of two populations within the same strain, that differ only in their percentage background genotype for the Booroola gene which increases fecundity.

The aim of this study was to determine whether the higher ovulation rate resulting from the Booroola fecundity gene is related to differences in mean peripheral FSH concentrations, or whether the differences in ovulation rate is determined primarily at the level of the ovaries.

3.2. Materials and Methods

3.2.1. Animals

Two different populations, either backcross or F₂, of Booroola Scottish Blackface ewes were used in this study (see section 2.1.1.2.). Briefly, the backcross population were 25% Booroola and 75% Scottish Blackface, whilst the background genotype in the F₂ population was 50% Booroola and 50% Scottish Blackface.

Backcross Booroola Scottish Blackface ewes, 2-4 years of age, were classified based on pedigree analysis as being either Fec^BFec⁺ (n=11) or Fec⁺Fec⁺ genotype (n=12) on the basis of at least 5 previous ovulation rate records. The average liveweight of ewes were 52.7 ± 2.1 and 55.5 ± 2.0kg for Fec^BFec⁺ and Fec⁺Fec⁺ respectively. These values were not significantly different and is in agreement with Bindon *et al.* (1982).

F₂ Booroola Scottish Blackface ewes, 2-4 years of age, were classified based on pedigree analysis as being either Fec^BFec^B (n=9) and Fec^BFec⁺ (n=9) or Fec⁺Fec⁺ genotype (n=9) on the basis of at least 5 previous

ovulation rate records. The average liveweight of ewes was 41.8 ± 2.2 , 39.3 ± 2.4 and 45.6 ± 2.0 kg for $Fec^B Fec^B$, $Fec^B Fec^+$ and $Fec^+ Fec^+$ respectively, which were not significantly different.

3.2.2. Experimental protocol

3.2.2.1. The backcross population during seasonal anoestrus (Experiment 1)

During late seasonal anoestrus (September) twice daily (at 09.00 and 16.00 hours) blood samples (10ml) were obtained by jugular venepuncture for 5 days, with the first day of sampling designated day 1. Hourly samples were also taken in this way for an additional 6 hours on day 2. All ewes were given a single injection of 750 i.u. i.m. human chorionic gonadotrophin, hCG (Chorulon, Intervet, Cambridge) on day 3, since this dose has previously been shown to induce ovulation in Scottish Blackface ewes during seasonal anoestrus (Webb *et al.*, 1992b). On day 5 the ewes underwent mid-ventral laparoscopy as described in section 2.2.2.

3.2.2.2. The F_2 population during seasonal anoestrus (Experiment 2)

During seasonal anoestrus (July), twice daily blood samples (10ml) were obtained by jugular venepuncture for 15 days, with hourly samples also being taken for 8 hours on day 11. On day 15, all ewes were given a single i.m. injection of 750 i.u. hCG. On day 17, the ewes underwent mid-ventral laparoscopy to assess ovulation rate as described previously (section 2.2.2.).

3.2.2.3. The backcross population during the oestrous cycle (Experiment 3)

During the breeding season (October) backcross Booroola Scottish Blackface ewes were run with a raddled ram and checked twice daily for oestrus. All ewes were sampled twice daily by jugular venepuncture from day -2 until day 10 of the next cycle (with day 0 being the day of behavioural oestrus). Hourly blood samples (10ml) were also collected for 8 hours (between 09.00 and 16.00 hours) on day -1 and again on day 7. Laparoscopy was performed on day 3 to determine ovulation rate. On day 8, all ewes received a single intramuscular injection of 750 i.u. hCG and laparoscopy was performed on day 10 to determine the subsequent, induced ovulation rate.

3.2.2.4. The F₂ population during the oestrous cycle (Experiment 4)

During the breeding season (November) F₂ Booroola Scottish Blackface ewes were run with a raddled ram and checked twice daily for oestrus. All ewes were sampled twice daily by jugular venepuncture from day -3 of the next cycle to day 13 (with day 0 being the day of behavioural oestrus). Blood samples from the jugular vein were collected hourly for an additional 6 hours during the mid-luteal phase (either day 6, 7 or 8). Laparoscopy was performed on all ewes during the luteal phase (days 9-12) to determine whether the ewes had ovulated and ovulation rate.

3.2.3. Hormone assays

3.2.3.1. FSH

The concentrations of FSH in peripheral serum samples were determined in duplicate using a previously described radioimmunoassay (section 2.3.2.). The minimum detectable value was 0.25ng/ml (n=8 assays). The inter-assay coefficient of variation (CV) was 9.8% and the intra-assay CV was 7.4%.

3.2.3.2. Progesterone

The concentrations of progesterone in peripheral serum were determined using a non-extracted radioimmunoassay (see section 2.3.4.). The minimum detectable value was 0.16ng/ml and the intra-assay CV was 8.8%. All the samples analysed were found to be less than the minimum detectable level (0.16ng/ml), confirming that the ewes were anoestrus.

3.2.3.3. Statistical analyses

FSH concentrations in the twice daily samples for each animal were expressed as an arithmetic mean. These means were then used to obtain a group arithmetic mean (\pm s.e.m.) for each day. All normally distributed data, as assessed from the Statworks package on the Apple Macintosh, are expressed as arithmetic means (\pm s.e.m.) and compared by Student's t test using the Minitab programme on the Apple Macintosh. Hourly samples for each ewe, within a genotype, were expressed as a group mean (\pm s.e.m.) for each hourly sample. Individual ovulation rates, within a season, were expressed as group arithmetic means (\pm s.e.m.) and compared by Student's t test. To compare between the breeding season and seasonal anoestrus, the individual difference between ovulation rates was calculated and expressed

as group arithmetic mean (\pm s.e.m.) and compared by Student's t test to see if the data were statistically significant.

3.3. Results

In all experiments, the influence of bodyweight upon FSH concentrations and ovulation rates was not statistically significant and was therefore excluded from further analyses.

3.3.1. Experiment 1

3.3.1.1. FSH

From blood samples that were collected hourly, mean peripheral FSH concentrations were significantly higher ($P < 0.001$) in heterozygous ewes compared to non-carrier ewes (Table 3.1.). However, mean peripheral FSH concentrations during seasonal anoestrus from blood samples collected twice daily did not differ between genotypes (Table 3.2.).

3.3.1.2. Ovulation rate

Nearly 83% of ewes ovulated after a single 750 i.u. i.m. injection of hCG. Three $Fec^B Fec^+$ ewes and one $Fec^+ Fec^+$ ewe failed to ovulate in response to the hCG treatment. Genotypic mean ovulation rates during seasonal anoestrus were not significantly different between genotypes ($Fec^B Fec^+$ ovulation rate: 1.73 and $Fec^+ Fec^+$ ovulation rate: 1.75) when observed at laparoscopy several days after hCG treatment.

3.3.2. Experiment 2

3.3.2.1. FSH

In blood samples collected hourly during seasonal anoestrus, mean peripheral FSH concentrations were significantly higher ($P < 0.05$) in

Table 3.1. FSH concentrations (ng/ml) in blood samples taken hourly during seasonal anoestrus (Experiment 1) and during the breeding season (Experiment 2) in two populations of Scottish Blackface ewes with and without the Fec^B gene.

Season	Population	Genotype		
		$Fec^B Fec^B$	$Fec^B Fec^+$	$Fec^+ Fec^+$
Seasonal anoestrus	backcross		2.50 ± 0.05^a (n=11)	2.26 ± 0.04^b (n=12)
Seasonal anoestrus	F ₂	2.68 ± 0.05^c	2.50 ± 0.03^d	2.56 ± 0.02^d
Oestrous cycle follicular phase	backcross		1.26 ± 0.11^e	1.15 ± 0.07^e
Oestrous cycle luteal phase	backcross		1.85 ± 0.05^f	1.94 ± 0.04^f
Oestrous cycle luteal phase	F ₂	3.51 ± 0.19^g	2.73 ± 0.08^h	2.43 ± 0.06^j

For each group, across rows, values with differing superscripts are significantly different

For the backcross population a versus b, $P<0.001$

For the F₂ population c versus d, $P<0.05$, g versus h, g versus j, $P<0.01$

Table 3.2. Mean (\pm s.e.m.) ovulation rate (following 750i.u. hCG) and mean (\pm s.e.m.) FSH concentrations (ng/ml), in blood samples taken twice daily, during seasonal anoestrus for the backcross (Experiment 1) and the F₂ populations (Experiment 2) of Scottish Blackface ewes with and without the Fec^B gene.

Seasonal Anoestrus	Population	Genotype		
		Fec ^B Fec ^B	Fec ^B Fec ⁺	Fec ⁺ Fec ⁺
Ovulation rate *	backcross		1.7 \pm 0.4 ^a (n=11)	1.8 \pm 0.3 ^a (n=12)
FSH concentration	backcross		2.3 \pm 0.2 ^b	2.4 \pm 0.2 ^b
Ovulation rate **	F ₂	2.7 \pm 0.6 ^a (n=9)	1.9 \pm 0.4 ^{a,b} (n=9)	0.9 \pm 0.3 ^b (n=9)
FSH concentration	F ₂	2.6 \pm 0.1 ^c	2.3 \pm 0.1 ^d	2.4 \pm 0.1 ^d

For each population, across rows, values with differing superscripts are significantly different

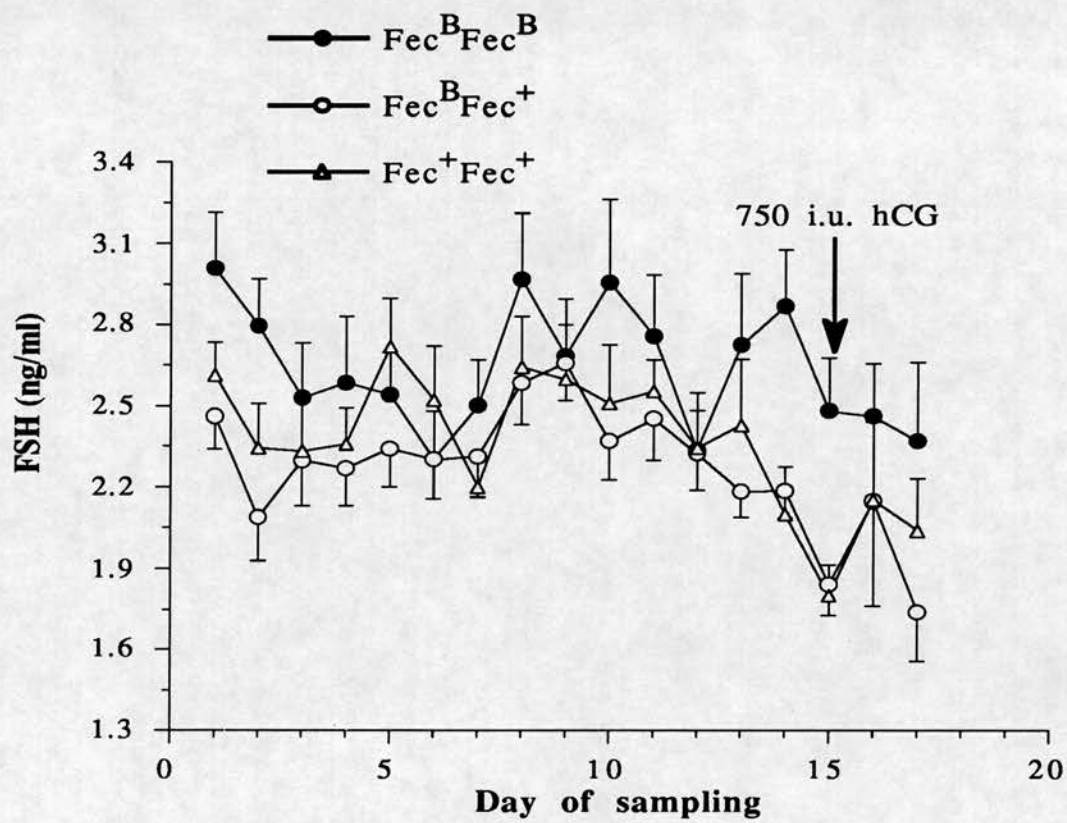
For the F₂ population a versus b, P<0.01, c versus d P<0.05

n = number of animals

* Mean ovulation rate from all ewes (following 750 i.u. hCG, 83% of ewes ovulated)

** Mean ovulation rate from all ewes (following 750 i.u. hCG, 78% of ewes ovulated)

Figure 3.1. Mean (\pm s.e.m.) serum FSH concentrations (ng/ml), from the three genotypes of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=9 per genotype) for 17 days during seasonal anoestrus. All ewes received a single i.m. injection of 750 i.u. hCG on day 15 (Experiment 2). Twice daily serum samples for individual ewes, from each genotype, are expressed as an arithmetic mean. These means were used to calculate a group arithmetic mean.



Fec^BFec^B ewes compared to the other two genotypes, which were similar (Table 3.1.). For each genotype, mean (\pm s.e.m.) serum FSH concentrations in samples collected twice daily for 17 days are summarized in Figure 3.1. and were significantly greater ($P < 0.05$) in Fec^BFec^B ewes compared to Fec^BFec⁺ and Fec⁺Fec⁺ ewes. The mean (\pm s.e.m.) peripheral FSH concentrations in samples collected twice daily did not differ between Fec^BFec⁺ and Fec⁺Fec⁺ ewes (Table 3.2.). Mean peripheral serum FSH concentrations for each genotype in samples collected twice daily after hCG treatment (days 15-17) were not significantly different from the mean (\pm s.e.m.) serum FSH concentrations for two days prior to treatment.

3.3.2.2. Ovulation rate

Nearly 77% of ewes ovulated after a single 750 i.u. im. injection of hCG. Four Fec⁺Fec⁺ ewes, two Fec^BFec⁺ ewes but no Fec^BFec^B ewes failed to ovulate. Mean ovulation rates for all ewes during seasonal anoestrus differed significantly ($P < 0.05$) between Fec^BFec^B ewes (ovulation rate: 2.7), Fec⁺Fec⁺ ewes (ovulation rate: 0.9), with Fec^BFec⁺ ewes (ovulation rate: 1.9) being intermediate (Table 3.2.).

3.3.3. Experiment 3

3.3.3.1. FSH

For each genotype, mean FSH concentrations in blood samples taken hourly were significantly lower ($P < 0.001$) on day -1 (follicular phase) of the oestrous cycle compared to day 7 (luteal phase) as shown in Table 3.1. During the oestrous cycle (day -1 or day 7) there was no significant difference between genotypes in mean peripheral FSH concentration from blood samples taken hourly (Table 3.1.). Mean serum FSH concentrations obtained in twice daily blood samples which were collected during the

oestrous cycle (day -1 to day 10) are shown in the **Figure 3.2. Upper panel** and were not significantly different between genotypes (**Table 3.3.**).

3.3.3.2. Ovulation rate

During the breeding season the mean ovulation rates differed significantly ($P < 0.001$) between genotypes ($Fec^B Fec^+$ ovulation rate: 3.5 and $Fec^+ Fec^+$ ovulation rate: 1.7). A single injection of hCG administered on day 8 of the cycle resulted in a 68% increase in the ovulation rate for each genotype so maintaining the genotypic difference ($P < 0.001$) in ovulation rate ($Fec^B Fec^+$ ovulation rate: 5.0 and $Fec^+ Fec^+$ ovulation rate: 2.5).

3.3.4. Seasonal comparisons in the backcross population

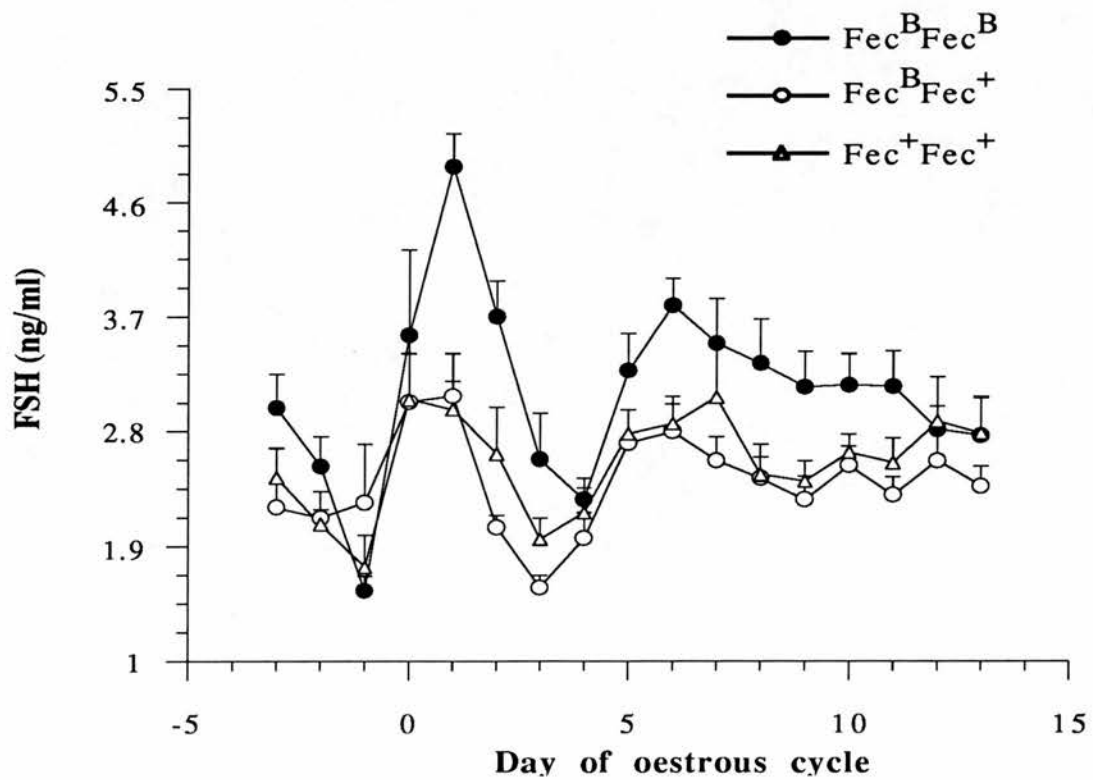
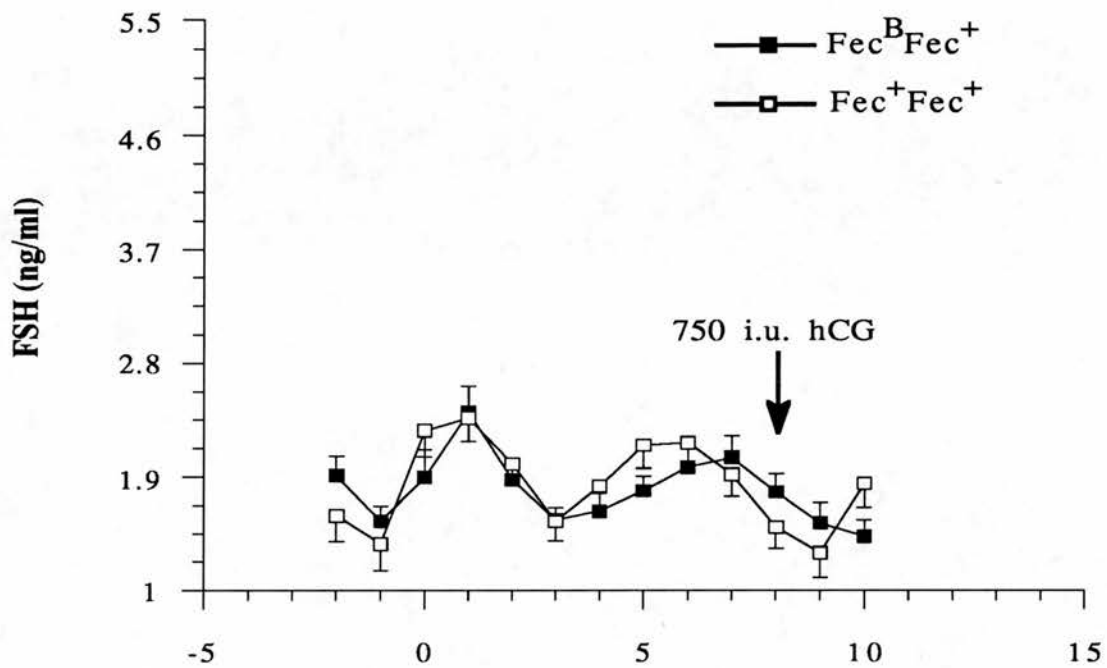
3.3.4.1. FSH

For each genotype, FSH concentrations in blood samples taken on an hourly basis were significantly greater ($P < 0.001$) during seasonal anoestrus when compared to those collected during the oestrous cycle (day -1 or day 7). FSH concentrations in blood samples taken twice daily were significantly greater ($P < 0.05$) during seasonal anoestrus compared to mean concentrations during the oestrous cycle for heterozygous ewes. No such seasonal difference existed in mean FSH concentration in blood samples taken twice daily from non-carriers of the Booroola fecundity gene.

3.3.4.2. Ovulation rate

Between seasons, the mean ovulation rate was found to be significantly lower ($P < 0.01$) during seasonal anoestrus in heterozygous ewes, whilst in non-carriers there was no seasonal difference in mean ovulation rate.

Figure 3.2. Upper panel Mean (\pm s.e.m.) serum FSH concentrations (ng/ml), from the two genotypes of the backcross population possessing the Booroola fecundity (Fec^B) gene (n=11 for heterozygotes and n=12 for non-carriers), from day -2 to day 10 of the oestrous cycle, with day 0 being the day of behavioural oestrus (Experiment 1). **Lower panel** Mean (\pm s.e.m.) serum FSH concentrations (ng/ml), from the three genotypes of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=9), from day -4 to day 13 of the oestrous cycle, with day 0 being the day of behavioural oestrus (Experiment 4). Twice daily serum samples for individual ewes, from each genotype, are expressed as an arithmetic mean. These daily within animal means were used to calculate a group arithmetic mean.



3.3.5. Experiment 4

3.3.5.1. FSH

Mean FSH concentrations in blood samples collected hourly during the luteal phase (day 6, 7 or 8) differed significantly ($P < 0.01$) between genotypes (Table 3.1.). For each genotype, mean (\pm s.e.m.) concentrations of FSH in samples collected twice daily from 3 days before to 13 days after oestrus (day 0) are shown in Figure 3.2. Lower panel. Mean serum FSH concentrations during the oestrous cycle were significantly greater ($P < 0.01$) in $Fec^B Fec^B$ ewes compared to the other two genotypes. Mean FSH concentrations were not significantly different between $Fec^B Fec^+$ and $Fec^+ Fec^+$ ewes (Table 3.3.).

3.3.5.2. Ovulation rate

During the breeding season the mean ovulation rates differed significantly ($P < 0.001$) between all three genotypes (Table 3.3.).

3.3.6. Seasonal comparisons in the F_2 population

3.3.6.1. FSH

For each genotype, FSH concentrations in blood samples collected on an hourly basis, were significantly greater ($P < 0.05$) during the luteal phase of the oestrous cycle (day 6, 7 or 8), compared to those collected in the non-breeding season. There was no seasonal difference in mean serum FSH concentrations from samples taken twice daily for $Fec^B Fec^+$ and $Fec^+ Fec^+$ ewes. Mean serum FSH concentrations from samples taken twice daily from $Fec^B Fec^B$ ewes were significantly ($P < 0.05$) elevated during the breeding season, compared to values during seasonal anoestrus.

Table 3.3. Mean (\pm s.e.m.) ovulation rate and mean (\pm s.e.m.) FSH concentration (ng/ml), in blood samples taken twice daily, during the oestrous cycle for the backcross (Experiment 3) and the F₂ populations (Experiment 4) in Scottish Blackface ewes with and without the Fec^B gene.

Oestrous cycle	Population	Genotype		
		Fec ^B Fec ^B	Fec ^B Fec ⁺	Fec ⁺ Fec ⁺
Ovulation rate	backcross		3.5 \pm 0.4 ^a (n=11)	1.7 \pm 0.1 ^b (n=12)
FSH concentration	backcross		1.8 \pm 0.1 ^b	1.8 \pm 0.1 ^b
Ovulation rate	F ₂	4.4 \pm 0.4 ^a (n=9)	2.4 \pm 0.2 ^b (n=9)	1.6 \pm 0.2 ^c (n=9)
FSH concentration	F ₂	3.1 \pm 0.2 ^d	2.4 \pm 0.1 ^e	2.6 \pm 0.1 ^e

For each population, across rows values with differing superscripts are significantly different

For the backcross population a versus b, P<0.001

For the F₂ population a versus b, b versus c and a versus c, P<0.001

d versus e, P<0.01

n = number of animals

3.3.6.2. Ovulation rate

There was no seasonal difference in ovulation rate for heterozygous and non-carriers of the Booroola fecundity gene. For homozygous ewes, the mean ovulation rate was significantly higher ($P < 0.05$) during the oestrous cycle compared to seasonal anoestrus.

3.4. Discussion

These studies indicate that differences in peripheral FSH concentrations are not wholly responsible for genotypic differences in ovulation rate. During seasonal anoestrus hCG-induced ovulation rate differences in the F_2 population were not observed between homozygous and heterozygous ewes despite significant differences in mean FSH concentration ($Fec^B Fec^B > Fec^B Fec^+$).

In agreement with other authors (Webb and Gauld, 1985b; Webb, 1988; Driancourt *et al.*, 1988; Webb *et al.*, 1989; Driancourt, Bodin, Boomarov, Thimonier and Elsen, 1990), our studies show that Booroola Scottish Blackface ewes can be induced to ovulate with a single challenge of hCG (750 i.u.) and the number of induced ovulations is normally representative of the genotype. This supports the hypothesis that the mechanism for controlling ovulation rate is functional throughout the year in the ewe (Webb *et al.*, 1985; Driancourt *et al.*, 1990). Interestingly, examination of mean peripheral FSH concentrations pre- and post-hCG treatment, in the F_2 population, revealed no significant change in FSH despite the final maturation and ovulation of follicles.

Although mean FSH concentrations for samples taken twice daily during the oestrous cycle in the F_2 population were significantly higher in the $Fec^B Fec^B$ line, compared to the other two lines, FSH concentrations did

not differ between the heterozygous and the homozygous non-carriers despite significant differences ($P < 0.05$) in breeding season ovulation rate. Similarly, in the backcross population, mean FSH concentrations in twice daily samples did not differ between the heterozygous and the homozygous non-carriers despite significant differences in breeding season ovulation rate ($P < 0.05$). It is interesting to note that genotypic differences in mean FSH concentration, which were observed by collecting twice daily samples, were not always apparent in samples which had been collected on an hourly basis. However, mean FSH concentrations were not significantly different between samples collected on an hourly basis and those collected from twice daily samples in any of the experiments performed in this study, indicating the variability of peripheral FSH concentrations and the need for multiple measurements.

The data suggest that the ovary may, in part, be responsible in controlling genotypic differences in ovulation rate, since the association of high FSH concentrations in genotypes with elevated ovulation rates is not always evident. The lack of association between mean FSH concentrations and ovulation rates was observed during the breeding season between heterozygous and non-carrier ewes, either from the F_2 or the backcross population. This comprehensive data set from $Fec^B Fec^+$ and $Fec^+ Fec^+$ ewes both extends and supports the results obtained by Driancourt (1990), whose evidence suggests that despite genotypic differences in ovulation rate, FSH concentrations did not differ between genotypes. During either seasonal anoestrus or in the oestrous cycle, FSH and ovulation rates are found to be higher in homozygous ewes compared to non-carriers of the gene, within the F_2 population, either on a mean daily or mean hourly basis. This association between elevated FSH concentrations and high ovulation rate is not always observed between

homozygous and heterozygous ewes, nor between heterozygous and non-carrier ewes (in both populations). It is at present unknown if possible genotypic differences exist in the underlying endogenous rhythm of peripheral FSH concentrations in Booroola Scottish Blackface ewes which can be investigated by removal of the negative feedback mechanisms through ovariectomy.

If the site of action of the major gene is at the level of the ovary, no genotypic differences in ovarian sensitivity to exogenous gonadotrophins have been found in terms of: response to FSH; FSH or LH binding characteristics to granulosa cells, LH receptor binding characteristics to thecal cells, the gonadotrophin sensitive components of the cellular cAMP generating system, or in LH induced cAMP and/or steroid synthesis (McNatty, Henderson, Fleming and Clarke, 1990a).

Analyses of the data showed no significant difference between genotypes in mean peripheral FSH concentration 72 hours prior to oestrus, that is, during the time of follicle recruitment (Driancourt *et al.*, 1985). Whilst gonadotrophins are required for growth of follicles, particularly those > 2mm in diameter (Dufour *et al.*, 1979; McNeilly *et al.*, 1986), differences in the pattern of gonadotrophin secretion within the physiological range may not be responsible for differences in ovulation rate. For example, it is not known whether differences in FSH are the cause or effect of differences in ovulation rate and caution is required to use associations between high FSH and high ovulation rate to conclude that the high FSH is responsible for the high ovulation rate (Driancourt, 1990). Two recent studies in hypophysectomized ewes, one in the Booroola Merino (Fry, Clarke, Cummins, Bindon and Cahill, 1988) and the other in Romanov and Ile de France ewes (Driancourt *et al.*, 1988) have demonstrated that breed and/or strain differences in ovulation rates could be maintained

when given the same amount of gonadotrophins. These studies indicated that high prolificacy may be due primarily to ovarian factors rather than pituitary gonadotrophins.

Whilst quantitative differences in peripheral FSH have been found, a qualitative difference in FSH may exist between genotypes. A number of isoforms have been identified which may be important in determining the biological potency of FSH (Robertson *et al.*, 1984). However, Robertson *et al.* (1984) found no genotypic differences between Booroola Merinos and control Merinos of a different strain on day 3 of the oestrous cycle. A more detailed study is required using suitable control animals to investigate the change in the relative proportions of the subunits of FSH between genotypes throughout the oestrous cycle.

The Booroola ewes in this study were approximately the same age, of similar bodyweight and received the same plane of nutrition so as to minimise non-genetic effects on ovulation rate (Scaramuzzi and Radford, 1983). It is known however, that there is substantial genetic variation between individuals such that, within a population, peripheral gonadotrophin concentrations may well vary simply due to chance variation in genes that were present in the foundation population. Care in comparing between genotypes must be exercised because the presence of genetic variation between families, the so-called 'sire effect', will exist which may be independent of the trait of interest. To avoid sire effects, the experimental groups from our studies were designed such that the progeny used were from many sires and therefore there was no requirement to consider the genetic bias of the groups by analysing the data using Restricted Maximum Likelihood (REML; Patterson and Thompson, 1971).

Current evidence indicates that the ovaries may have a more important role in the control of ovulation rate in the sheep than originally thought. Certainly it has been shown that FSH acts synergistically with IGF-1 to stimulate granulosa cell proliferation (Webb *et al.*, 1991). Hence synergism between gonadotrophins and other peripheral hormones and/or ovarian growth factors, may be of central importance. From the analyses of ovine follicular growth patterns, it appears that high ovulation rates can be achieved through different mechanisms (Driancourt *et al.*, 1985; Driancourt *et al.*, 1986; McNatty *et al.*, 1990). The characterisation of these mechanisms and demonstration of their importance can be achieved by studying in more detail the control of follicular growth especially in breeds which differ in prolificacy such as the Booroola Scottish Blackface ewe.

In conclusion, a single injection of hCG can induce follicles to ovulate either in the luteal phase of the oestrous cycle (Day 8) or during seasonal anoestrus, showing that the mechanisms controlling ovulation rate are still present during these times. However, FSH was found not to be wholly responsible for the genotypic differences in ovulation rate.

CHAPTER FOUR

Possible genotypic differences in FSH, before and after ovariectomy

4.1. Introduction

It is possible that the increased ovulation rate seen in ewes possessing the Booroola fecundity gene (Chapter 3) may, in part, be a result of a lower sensitivity of the hypothalamus/pituitary gland to the negative feedback effects of ovarian hormones, such as oestradiol or inhibin (section 1.2.3.). Higher concentrations of ovarian hormones need not therefore result in a greater reduction in the release of gonadotrophins (Land, 1976), hence there could be differences in ovulation rate without differences in the peripheral levels of gonadotrophins. If the Land hypothesis is correct, differences in gonadotrophins should be observed in high or low prolific breeds or strains after removal of the inhibitory actions of ovarian steroids, such as seen after long-term ovariectomy between Finnish landrace and Scottish Blackface ewes (Webb and Gauld, 1985a).

Studies which have investigated the effect of ovariectomy in ewes possessing the Booroola fecundity gene have provided conflicting evidence, with some authors reporting persistent Fec^B gene effects following ovariectomy (McNatty *et al.*, 1990a) and other authors finding no such effect (Bindon and Hillard, unpublished - see McNatty *et al.*, 1990a for review; Driancourt and Fry, 1990; McNatty, Hudson, Collins, Fisher, Heath and Henderson, 1989a; McNatty, Fisher, Collins, Hudson, Heath, Ball, Hudson, 1989). Possible reasons for this conflicting evidence could be due to the comparison of Booroola Merinos with Merinos of a different strain, the use

of animals which were of an unknown genetic background and/or the use of sibling animals which could therefore bias the results due to the presence of sire effects (see Chapter 3). As far as we are aware, this investigation is the first to be conducted with animals that are of known genetic background and have been selected from defined breeding lines in order to avoid the spurious effects that can be caused by the use of siblings and/or inappropriate controls.

The aim of this work was to investigate possible genotypic differences in the underlying endogenous release of peripheral FSH concentrations, which can be observed when the negative feedback sources are removed through ovariectomy, in two populations of ewes which differ in the percentage background genotype of the Booroola fecundity (Fec^B) gene.

4.2. Materials and Methods

4.2.1. Animals

The backcross Booroola Scottish Blackface ewes used in this study were the same animals ($Fec^B Fec^+$ $n=11$; $Fec^+ Fec^+$ $n=12$) as in Chapter 3 (see section 3.2.1.). This study (Experiment 1) was started in November several weeks after the study reported in the previous Chapter.

F_2 Booroola Scottish Blackface ewes 2-4 years of age were classified as being the $Fec^B Fec^B$ ($n=10$), $Fec^B Fec^+$ ($n=8$) and $Fec^+ Fec^+$ genotype ($n=8$) on the basis of at least 5 previous ovulation rate records. The average liveweight of ewes were 44.7 ± 3.7 , 47.4 ± 2.0 and 49.6 ± 1.5 kg for $Fec^B Fec^B$, $Fec^B Fec^+$ and $Fec^+ Fec^+$ respectively, which were not significantly different in agreement with Bindon *et al.* (1982).

4.2.2. Experimental protocol

4.2.2.1. Ovulation rate and FSH in the backcross population (Experiment 1)

During the breeding season (December) the oestrus of ewes was synchronized with a single prostaglandin (Estrumate, Coopers Animal Health Ltd.) injection (0.4ml Estrumate dissolved in 1.6ml of saline), run with a raddled ram and monitored twice daily for signs of oestrus (day 0). All ewes were sampled twice daily (at 09.00. and 16.00 hours) by jugular venepuncture from day 4 following synchronised oestrus up to day 7. On day 6, hourly jugular venepuncture samples were collected for 8 hours and on day 8 the ewes were ovariectomized (see section 2.2.3.). Twice daily blood sampling by jugular venepuncture was continued for 14 days post-ovariectomy with additional hourly samples being collected for 6 hours on day 13.

4.2.2.2. Ovulation rate and FSH in the F₂ population (Experiment 2)

During the breeding season (December) F₂ Booroola Scottish Blackface ewes were synchronised using vaginal progestagen sponges (Veramix, Crowley) for 12 days. All ewes were run with a raddled ram and checked twice daily for signs of oestrus (day 0). The ewes were sampled twice daily by jugular venepuncture from the first day of sponge removal (day -2) until day 7. On day 7, all ewes were ovariectomized as described previously in section 2.2.3, the day of ovariectomy was now termed day 0. Twice daily blood sampling by jugular venepuncture was continued for 14 days post-ovariectomy.

4.2.3. Hormone assays

4.2.3.1. FSH

Concentrations of FSH in peripheral serum samples were determined by radioimmunoassay (section 2.3.2.). The minimum detectable value was 0.97ng/ml. The inter-assay CV, using six standard quality control samples, was 12.2% and the intra-assay CV was 7.5%.

4.2.3.2. Progesterone

Concentrations of progesterone in peripheral serum were determined by radioimmunoassay (section 2.3.4.). The minimum detectable value was 0.1ng/ml and the intra-assay and inter-assay CV were 6.8% and 12.8% respectively.

4.2.3.3. Statistical analyses

FSH concentrations in the twice daily samples from each animal are expressed as an arithmetic mean. These means were then used to obtain a group arithmetic mean (\pm s.e.m.) for each day as in Chapter 3. All normally distributed data, as assessed from the Statworks package on the Apple Macintosh, are expressed as arithmetic means (\pm s.e.m.) and compared by Student's t test (using the Minitab programme on the Apple Macintosh). Samples collected hourly from each ewe, within a genotype, are expressed as a group mean (\pm s.e.m.). Individual ovulation rates, within a season, were expressed as a group arithmetic mean (\pm s.e.m.) and compared by Student's t test.

4.3. Results

In all experiments, the influence of bodyweight upon FSH concentrations and ovulation rates was not statistically significant and was therefore excluded from further analyses.

4.3.1. Experiment 1

4.3.1.1. Progesterone

Mean (\pm s.e.m) progesterone concentrations in once daily blood samples, taken from day 4-8 of the oestrous cycle, did not differ between heterozygous and non-carrier ewes (0.56 ± 0.31 and 0.47 ± 0.17 ng/ml respectively). Mean progesterone concentrations post-ovariectomy were not significantly above the minimum detectable limit of the assay.

4.3.1.2. FSH

From blood samples collected hourly, mean peripheral FSH concentrations during the luteal phase (day 6), which was prior to ovariectomy, were significantly elevated ($P < 0.05$) in non-carrier ewes compared to heterozygous ewes (Table 4.1.). However, 6 days after ovariectomy, no such genotypic difference in mean FSH concentrations, in blood samples which were collected hourly, was observed (Table 4.1.). Within genotype, FSH concentrations in blood samples which were collected hourly were significantly elevated ($P < 0.001$) after gonadectomy (Table 4.1.).

Table 4.1. Mean FSH concentrations (ng/ml \pm s.e.m.) in blood samples collected hourly pre- and post-ovariectomy in Scottish Blackface ewes with and without the the Fec^B gene.

Group	Population	Genotype	
		Fec ^B Fec ⁺	Fec ⁺ Fec ⁺
Pre-ovariectomy	backcross	2.61 \pm 0.04 ^a	3.01 \pm 0.07 ^b
Post-ovariectomy	backcross	7.97 \pm 0.11 ^c	8.00 \pm 0.07 ^c

Across rows values with differing superscripts are significantly different (P<0.05)

Pre-ovariectomy versus post-ovariectomy mean FSH concentrations for each genotype (P<0.001)

4.3.1.4. Pre-ovariectomy FSH

Mean peripheral FSH concentrations pre-ovariectomy (**Figure 4.1. Upper panel**), from blood samples collected twice daily, did not differ between genotypes (**Table 4.2.**).

4.3.1.3. Post-ovariectomy FSH

Mean peripheral FSH concentrations post-ovariectomy in blood samples collected twice daily (**Figure 4.1. Upper panel**) did not differ between genotypes (**Table 4.2.**). Within genotype, FSH concentrations from blood samples collected twice daily were significantly elevated ($P < 0.001$) after gonadectomy (**Table 4.2.**).

4.3.1.5. Ovulation rate

The mean ovulation rate as observed at surgery on the day of ovariectomy was significantly greater ($P < 0.05$) in heterozygous ewes compared to the non-carriers (**Table 4.2.**).

4.3.2. Experiment 2

4.3.2.1. Progesterone

The mean (\pm s.e.m) progesterone concentration (ng/ml) in a once daily blood sample, for each genotype, are shown in **Figure 4.2.** Individual profiles of mean (\pm s.e.m) progesterone concentrations (ng/ml) were also examined to ensure that each animal was in the luteal phase of the cycle, that is, exhibited progesterone concentrations of greater than 0.5ng/ml (section 1.3.2.).

Table 4.2. Mean ovulation rate (\pm s.e.m.) and mean FSH concentrations (ng/ml; \pm s.e.m.) in blood samples collected twice daily pre- and post-ovariectomy in Scottish Blackface ewes with and without the the Fec^B gene.

Group	Population	Genotype		
		$Fec^B Fec^B$	$Fec^B Fec^+$	$Fec^+ Fec^+$
Ovulation rate	backcross	-----	3.3 ± 0.5^a (n=11)	1.7 ± 0.2^b (n=12)
Pre-ovariectomy FSH	backcross	-----	2.9 ± 0.4^a	2.7 ± 0.2^a
Post-ovariectomy FSH	backcross	-----	7.7 ± 0.2^b	8.0 ± 0.2^b
Ovulation rate	F ₂	3.6 ± 0.7^a (n=10)	2.3 ± 0.2^b (n=8)	1.3 ± 0.2^c (n=8)
Pre-ovariectomy FSH	F ₂	3.0 ± 0.2^c	2.2 ± 0.2^d	2.5 ± 0.4^d
Post-ovariectomy FSH	F ₂	10.4 ± 0.5^e	8.6 ± 0.3^f	8.7 ± 0.4^f

Across rows values with differing superscripts are significantly different
Ovulation rate, a versus b, $P < 0.05$, a verses c, b verses c, $P < 0.01$
FSH concentrations pre-ovariectomy compared to post-ovariectomy for
each genotype are significantly different ($P < 0.001$)

4.3.2.2. Pre-ovariectomy FSH

Mean peripheral FSH concentrations in blood samples, collected twice daily from day -2 to day 7 (**Figure 4.1. Lower panel**), were significantly greater ($P < 0.05$) in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ ewes compared to the other two genotypes (**Table 4.2.**). However, the mean peripheral FSH concentrations were not significantly different between $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$ ewes (**Table 4.2.**).

4.3.2.3. Post-ovariectomy FSH

Mean serum FSH concentrations in twice daily blood samples were significantly greater ($P < 0.001$) in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ ewes compared to $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$ ewes (**Table 4.2.**). However, the mean serum FSH concentrations were not significantly different between $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$ ewes (**Table 4.2.**). Within each genotype following ovariectomy, mean peripheral FSH concentrations (**Figure 4.1. Lower panel**) were significantly ($P < 0.001$) elevated.

4.3.2.4. Ovulation rate

On the day of ovariectomy (day 7), mean ovulation rates were significantly different between $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$, $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$ ewes (**Table 4.3.**).

4.4. Discussion

These data show no consistent association between ovulation rate and pre-ovariectomy FSH concentration for two populations of the Booroola genotypes. Within the F_2 population, despite no significant differences in ovulation rate between $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ and $\text{Fec}^{\text{B}}\text{Fec}^{+}$ ewes, mean pre-ovariectomy FSH concentrations in twice daily blood samples did differ between these two

Figure 4.1. Upper panel (Experiment 1). Mean (\pm s.e.m.) serum FSH concentrations (ng/ml), in the two genotypes of the backcross population possessing the Booroola fecundity (Fec^B) gene (n=11 for heterozygous ewes and n=12 for non-carriers). Day 0 is the day of ovariectomy (equivalent to day 8 of the oestrous cycle). Lower panel (Experiment 2). Mean (\pm s.e.m.) serum FSH concentrations (ng/ml), in the three genotypes of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=8). Day 0 is the day of ovariectomy (equivalent to day 7 of the oestrous cycle). Twice daily serum samples in individual ewes from each genotype are expressed as an arithmetic mean. These daily within animal means were used to calculate a group arithmetic mean.

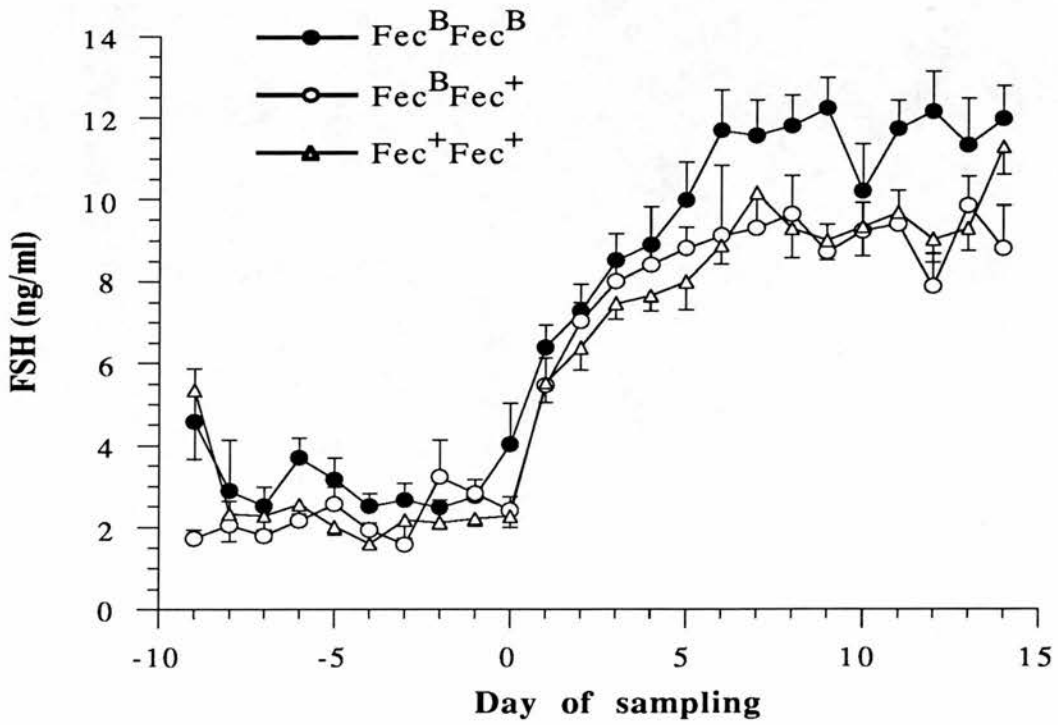
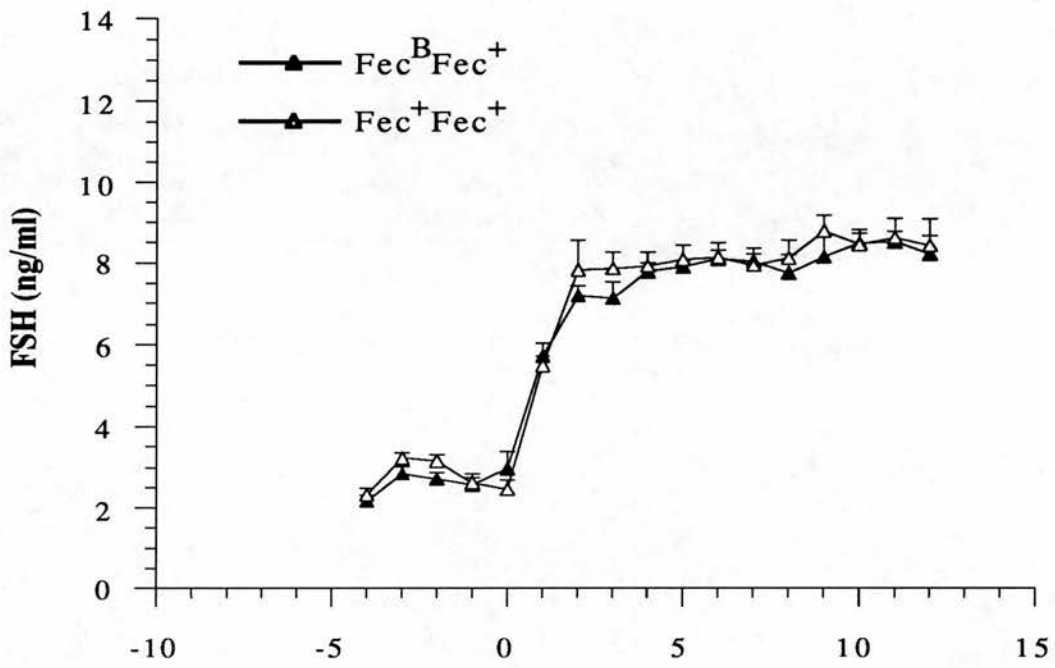
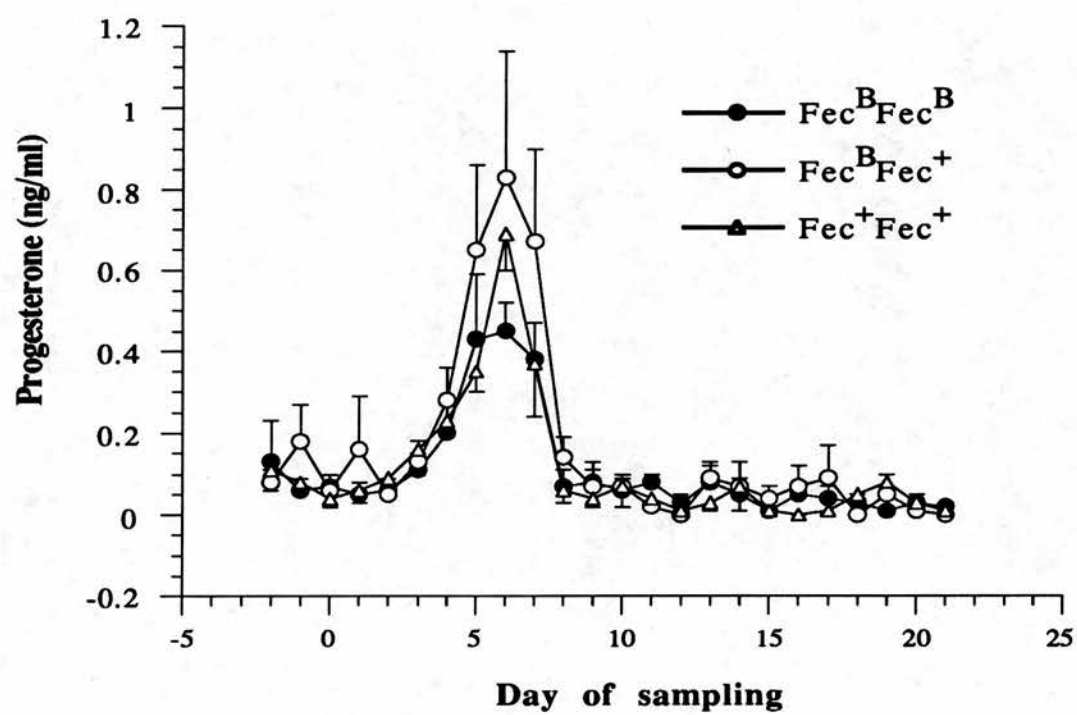


Figure 4.2. Mean (\pm s.e.m.) serum progesterone concentrations (ng/ml), from the three genotypes of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=8 per genotype). Day 0 being the day of ovariectomy on to day 7 of the oestrous cycle (Experiment 2). Once daily serum samples for individual ewes, from each genotype, are expressed as an arithmetic mean. These daily within animal means were used to calculate a group arithmetic mean.



genotypes ($P < 0.05$). Within the backcross population, $Fec^B Fec^+$ ewes had a greater mean ovulation rate ($P < 0.05$) than non-carriers of the Booroola fecundity gene, but again there was no genotypic difference in peripheral FSH concentration. Ovulation rates and FSH levels in the F_2 population were higher ($P < 0.05$) in ewes possessing the Booroola fecundity gene compared to non-carriers. In this study, synchronisation of ewes using either progestagen intra-vaginal sponges (F_2 population), or prostaglandin injection (backcross population) did not affect the normal breeding season ovulation rate expected for each genotype (Chapter 3).

Withdrawal of ovarian steroids by removal of the ovaries in the ewe results in profound chronic and time dependent changes in gonadotrophins (Martin, 1984; Webb and Gauld, 1985a). The effect of ovariectomy on peripheral LH concentrations has been well documented, showing an initial increase in LH pulse frequency and a rise in peripheral LH concentrations which exhibit a circroral pattern (Diekman and Malven, 1973; Davis and Borger, 1974; Butler, Malven, Willett and Bolt, 1972; Joseph, Currie and Rawlings, 1992). The increase in LH pulse frequency in the ovariectomized animal is presumably preceded by an increase in GnRH pulse frequency. The relative increases in LH and FSH after ovariectomy are reported to be of similar magnitude within a population of sheep (Bolt, 1981). In this study, ovariectomy resulted in a rapid rise in FSH concentration, which lasted for several days, as a result of the removal of the negative feedback effects of the ovarian hormones (see Goodman and Karsch, 1981 for review; Driancourt, Fry, Clarke and Cahill, 1987). This rapid rate of increase in FSH concentration is thought to be independent of season (Montgomery, Martin, Blanc and Pelletier, 1987; Joseph *et al*, 1992) and in the rat is mediated by both GnRH-dependent and GnRH-independent mechanisms (Berardo and Depaolo, 1986).

For either population, there were no significant genotypic differences in the time taken for FSH to reach a plateau indicating no genotypic differences in the response time taken for the pituitary and hypothalamus glands to respond to the removal of the negative feedback hormones from the ovary. In the F₂ population, the genotypic differences in FSH concentration that exist prior to ovariectomy, between homozygous carriers and the other two genotypes, persist after ovariectomy. This suggests that genotypic differences do exist in the release of FSH at the level of the hypothalamus and/or pituitary gland. However, there were no genotypic differences in the FSH concentration both prior to, or after, ovariectomy for heterozygous ewes compared to the non-carrier ewes within either population. This is of particular interest since it shows that the pituitary gland and/or the hypothalamic gland from heterozygous and non-carrier ewes release similar concentrations of FSH and yet ovulation rate differences are maintained in the intact animal.

Within either population, the ovary may, in part, be responsible for controlling genotypic differences in ovulation rate despite the similar peripheral FSH concentrations observed between heterozygous and non-carrier ewes. McNatty *et al.* (1989b) concluded that the increase in ovulation rate between homozygous and non-carrier ewes was unlikely to be due to gene specific differences in the sensitivity of the hypothalamic-pituitary axis to ovarian hormones. However heterozygotes and non-carriers were not included in the study by McNatty *et al.* (1989b) and furthermore care must be exercised when comparing genotypes of unknown genetic background as discussed previously.

It may be possible that homozygous ewes, compared to the other two genotypes, achieve higher ovulation rates through a different mechanism

which involves elevated FSH concentrations. However, the possibility that genotypic differences in follicular responsiveness or sensitivity to FSH cannot be excluded. In conclusion, this study found no genotypic differences between heterozygous and non-carrier ewes, from either population, in the underlying pattern of FSH release when the negative feedback effects of ovarian hormones are removed through ovariectomy. During the oestrous cycle (day -2 to day 7), genotypic differences in FSH concentration, between homozygous carriers compared to either of the other two genotypes, were also present after ovariectomy in the F₂ population providing evidence for genotypic differences in pituitary gland release of FSH.

CHAPTER FIVE

Validation an *in vitro* culture system

5.1. Introduction

The follicle is the functional unit of the ovary, providing a micro-environment for the oocyte and producing steroids (Torrance, Telfer and Gosden, 1989; Seamark, Moor and McIntosh, 1974). A detailed understanding of the effect of peripheral hormones and/or growth factors on growth and development of the follicle has been investigated using whole follicle *in vitro* culture techniques for the mouse (Torrance *et al.*, 1989; Carroll, Wood and Whittingham, 1989; Ryle, 1972), hamster (Roy and Greenwald, 1985; Roy and Greenwald, 1988), pig (Greenwald and Moor, 1989; Hirao, Miyano and Kato, 1991), bovine (Taha and Schellander, 1991; Hulshof, Bevers, Van der Donk and Van den Hurk, 1991) and sheep (Moor, 1973; Moor, Hay, McIntosh and Caldwell, 1973).

It was decided to develop a culture procedure for small ovine follicles from 200-800 μ m in diameter since *in vivo* evidence suggests that follicles within this size range have maximum growth rates whilst rates of atresia are virtually zero (Turnbull *et al.*, 1977). In this study, a follicle was classified as 'growing' if the diameter increased above the limit of detection of the graticule (10 μ m). This 'growth' may be due to an increase in the number of cells within the follicle, or due to an increase in accumulation of follicular fluid.

Individual mice follicles can now be grown *in vitro* from preantral through to Graafian stages (Nayudu and Osborn, 1992). The *in vitro* culture procedure employed by these authors was suitable for the measurement of follicular growth and oestradiol production in response to FSH stimulation.

The modification of this culture technique may provide a method to enable measurement of genotypic differences in response of FSH for small antral follicles in the Booroola Scottish Blackface ewe.

The aim of this study, to validate an *in vitro* culture system for small antral ovine follicles in order to test the hypothesis that the Booroola fecundity gene expresses its effects in ewes by influencing follicular responsiveness to FSH.

5.2. Materials and Methods

5.2.1. Animals

The animals used in Experiments 1 and 2 were Scottish Blackface ewes of approximately 2-4 years of age.

The F₃ Booroola Scottish Blackface lambs used in Experiment 3 were obtained from breeding either F₂ homozygous carriers or F₂ homozygous non-carriers of the Booroola fecundity gene. The F₃ lambs were approximately 6-12 months of age and had been assigned to either the Fec^BFec^B or Fec⁺Fec⁺ genotype on the basis of their sire/dam genotype (see section 2.1.1.2.). Lamb ovaries were chosen in preference to adult material since it was easier to dissect the follicles from the stromal tissue as it seemed to be less fibrous than from older animals. The animals were initially kept at Blythbank, which is a traditional upland grassland farm, before being transferred to Dryden farm at least 4 weeks prior to the start of experimentation.

5.2.2. Experimental protocol

5.2.2.1. Effect of FSH on follicle growth and oestradiol production (Experiment 1)

The aim of Experiment 1 was to determine the dose of FSH which would result in optimal follicular development. The treatment groups involved incubation of the follicles in 200 μ l of α MEM (see section 2.5.), supplemented with FSH (NIAMDD-oFSH-14) either 2.5 μ g/ml, 5.0 μ g/ml or 7.5 μ g/ml (500, 1,000 or 1,500ng/well respectively). The doses of FSH chosen for this experiment were thought to be potentially capable of inducing follicular growth.

During seasonal anoestrus (May) one Scottish Blackface ewe was ovariectomized each day as described in section 2.2.3. The absence of a corpus luteum was evidence that the ewe was in anoestrus. The procedure for the *in vitro* culture of the follicles is described in section 2.5. Follicles which were of approximately the same visual condition and size (section 2.5.), were randomly assigned to one of several treatment groups. Follicles were cultured from day 0, the first day of culture, until day 4. The culture media was changed and collected on day 2 and collected on day 4.

5.2.2.2. Effect of the luteal phase on *in vitro* growth and oestradiol production (Experiment 2)

The aim of Experiment 2 was to investigate possible differences in growth and oestradiol production of follicles that were explanted on different days of the luteal phase. During the breeding season (March) 8 Scottish Blackface ewes were run with a raddled ram and monitored twice daily for signs of oestrus. All ewes were synchronised with a single prostaglandin (Estrumate, Coopers Animal Health Ltd, UK) injection (0.4ml

Estrumate dissolved in 1.6ml saline). Ovariectomy was performed (see section 2.2.3.) on 2 ewes per day, either on day 7, 9 or 11 of the oestrous cycle. The follicles were incubated in 200 μ l of culture media (section 5.2.2.1.) which was supplemented with 2.5 μ g/ml FSH (500ng/well) as described in section 2.5. Follicles were cultured from day 0, the first day of culture, until day 4. The culture media was changed and collected on day 2 and collected on day 4.

5.2.2.3. Effect of daily removal of follicles on growth and oestradiol production (Experiment 3)

The aims of this study were, firstly to investigate whether daily (temporary) removal from the incubator did not adversely affect the development of the follicles, which had been pre-incubated in either gassed or ungassed α MEM. Secondly, to investigate possible genotypic differences in follicular growth and oestradiol production response in response to FSH. Follicles were obtained from ewe lambs which were either homozygous carriers or non-carriers of the Booroola fecundity gene. The age at which puberty occurs in ewe lambs is markedly affected by the time of year and can vary both within and between breeds and can be affected by body weight of the animal, such that the onset of puberty can range between 6-16 months (Robertson, 1977). The lambs used in this experiment were 6 months old and so each ovary was examined for signs of corpora lutea, although none were found.

During November, one F₂ Booroola Scottish Blackface lamb was ovariectomized per day and the procedure for *in vitro* culture followed as described in section 2.5. The follicles were incubated in 150 μ l culture media, supplemented with 2.5 μ g/ml FSH (375ng/well) and only differed from that described in section 5.2.2.1. by the addition of 1% pyruvate,

which may be important metabolic substrate (Spears and Boland, personal communication; Boland, Humpherson, Leese and Gosden, 1993). A reduced volume of culture media was used to try to concentrate the low levels of oestradiol produced by the follicles. Follicles were cultured from day 0 to day 3, with the media being changed and collected on day 2 and collected on day 3. Follicles were not cultured from day 0 to day 4 as previously described since results from the previous two experiments demonstrated that the numbers which were still growing by day 4 was low.

The treatment groups involved the use of two separate desiccators. Follicles from each genotype were incubated either inside one desiccator (termed desiccator 1) and examined only at the end of the culture period or incubated inside a separate desiccator (termed desiccator 2) and examined daily. During this investigation, the follicles from both genotypes were preincubated in one type of holding media (α MEM supplemented with 25mM sodium hydrogen carbonate and 5% lamb serum at 37°C), which was either continuously gassed with 5% carbon dioxide in air, or not gassed with 5% carbon dioxide in air.

5.2.3. *Hormone assays*

5.2.3.1. Oestradiol

Concentrations of oestradiol in culture media, α MEM pre-incubation media and Leibovitz-L15 dissection media, were determined. Alpha MEM contains 10mg/ml Phenol red (Imperial Labs. information Sheets, 1989) which act as a weak oestrogen (Ernst, Schmid and Froesch, 1989; Ortmann, Sturm, Knuppen and Emons, 1990) and consequently 'blank' samples of pre-incubation and culture media were routinely assayed. The concentration of oestrogen in 'blank' media samples was below the minimum detectable level

of the assay (19.2pg/ml). The inter-assay CV was 9.3% and the intra-assay CV was 8.1%.

5.3.2.2. Statistical analyses

Normally distributed data, as assessed by the Statworks package on the Apple Macintosh, are expressed as arithmetic means (\pm s.e.m.) and compared either by Students' t test or by analysis of covariance, to allow for multiple measurements, using the Minitab programme on the Apple Macintosh (see section 2.6.). Data which was not normally distributed was logarithmically transformed and statistically analysed before being presented as geometric means (\pm s.e.m.).

5.3. Results

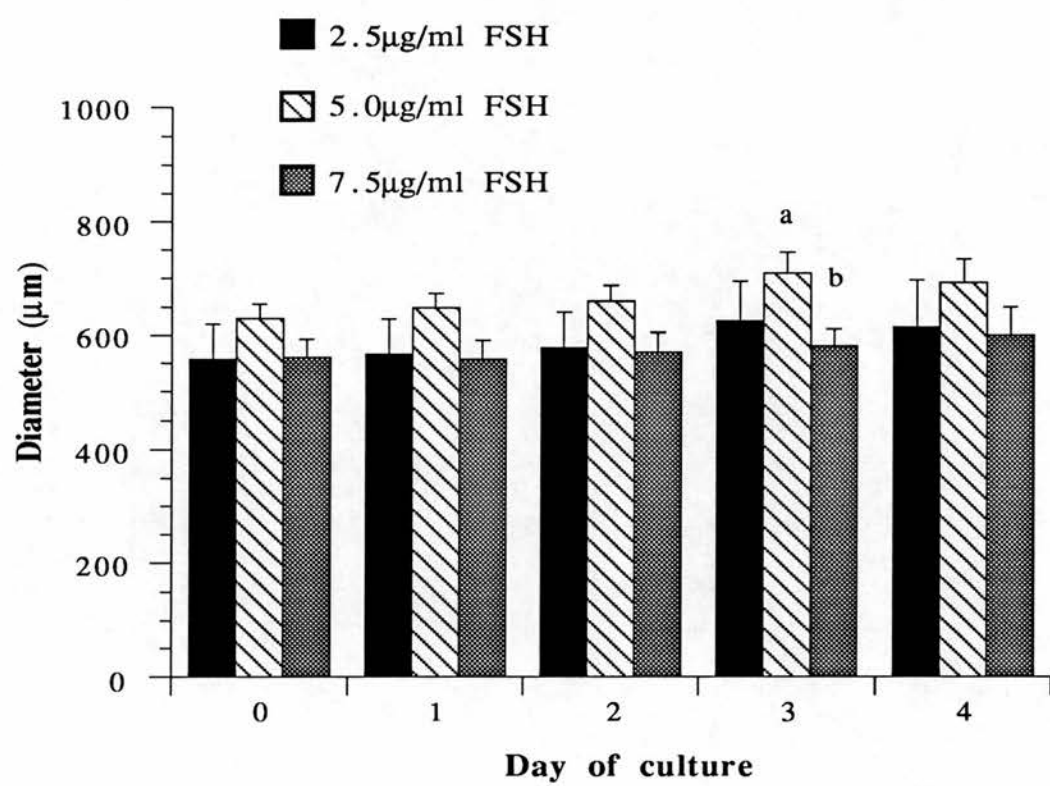
5.3.1. Experiment 1

5.3.1.1. Size of all follicles

The follicles obtained after ovarian dissection had diameters ranging in size between 200-800 μ m. In order to prevent a biased data set, follicles were randomized, on the basis of size and condition (see section 2.5.) into the various treatment groups such that the mean size (diameter) of all follicles on the first day of culture (day 0) did not differ between treatment groups (**Figure 5.1.**).

There was no significant correlation between the mean size of all follicles on day 0 and the change in size between days 0-2 of culture. There was no significant effect of FSH on mean (\pm s.e.m.) size (diameter) for all follicles on days 1, 2 or 4 (**Figure 5.1.**). On day 3 of culture, the mean diameter of all follicles incubated with 5 μ g/ml FSH, was significantly greater ($P < 0.05$) than those incubated with 7.5 μ g/ml FSH.

Figure 5.1. The effect of different doses of FSH *in vitro* upon the mean (\pm s.e.m.) diameter (μm) of all follicles dissected from the ovaries of Scottish Blackface ewes during seasonal anoestrus (Experiment 1). In the figure, the columns indicated by the a and b differed significantly ($P < 0.01$).



5.3.1.2. Size of growing follicles

The proportion of follicles which increased in size during *in vitro* incubation (approximately 39%) was not significantly affected by dose of FSH used or length of time in culture. For growing follicles, there was no strong linear relationship ($r < 0.5$) between size, either on day 0 or day 2, and follicular growth between days 0-2 or between days 2-4 respectively.

Mean (\pm s.e.m.) daily increase in size (%) for growing follicles, was not significantly affected by dose of FSH, except for follicles growing between days 2-3 compared with days 3-4, when incubated with $2.5\mu\text{g/ml}$ FSH (Figure 5.2.). In this group, there was a significant reduction ($P < 0.05$) in growth between days 2-3 compared with days 3-4. In addition, the percentage increase in size between days 2-3 by follicles incubated with $2.5\mu\text{g/ml}$ FSH was also significantly greater ($P < 0.05$) than by follicles incubated with $7.5\mu\text{g/ml}$ FSH.

5.3.1.3. Oestradiol production by all follicles

Irrespective of dose of FSH, there was no significant correlation between follicular size, either on day 2 or day 4, and geometric mean oestradiol production between days 0-2 or between days 2-4 respectively. For all cultured follicles, there was no strong linear relationship (r was < 0.5) between the change in follicular size and geometric mean oestradiol production between days 0-2 or between days 2-4 of culture.

Overall follicles, there was no significant effect of FSH on geometric mean oestradiol production between days 0-2 or between days 2-4 of culture (Figure 5.3.).

Figure 5.2. The effect of different doses of FSH *in vitro* upon mean daily increase in size (%) of growing follicles dissected from the ovaries of Scottish Blackface ewes during seasonal anoestrus (Experiment 1). In the figure, the columns indicated by the different letters differed significantly ($P < 0.05$)

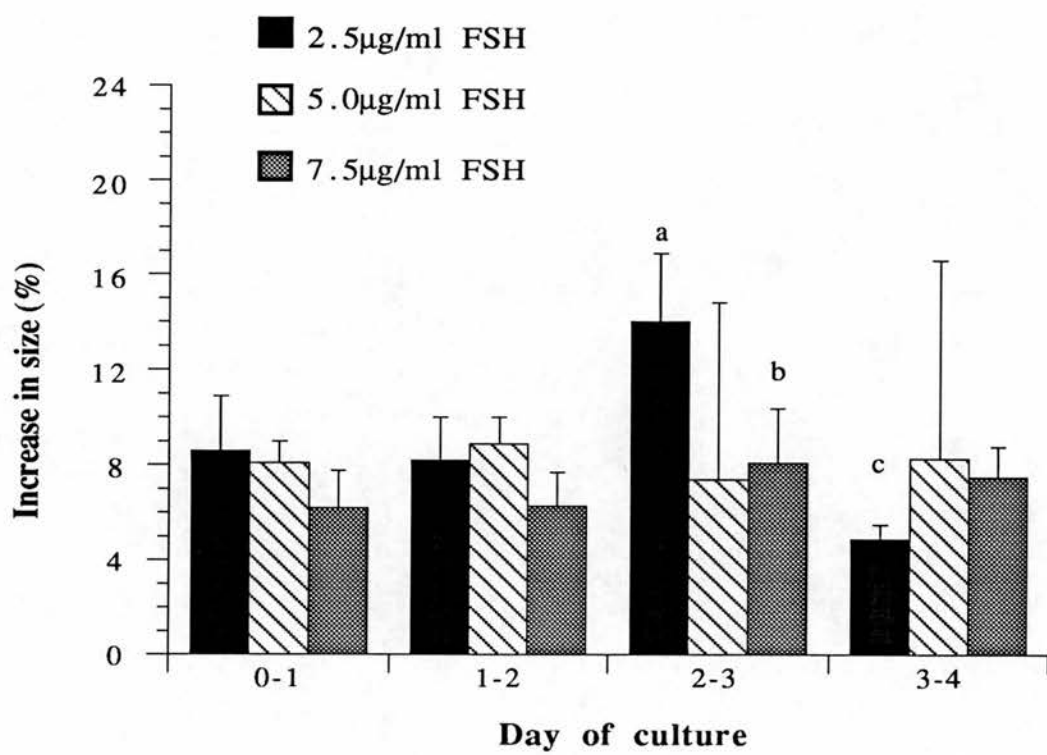
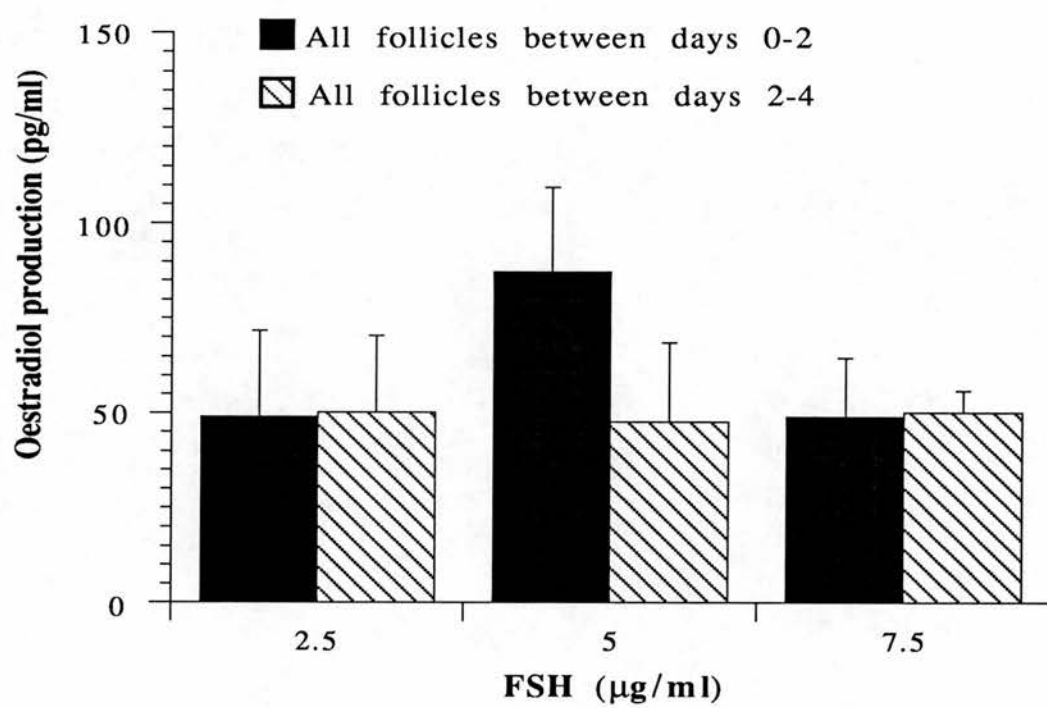


Figure 5.3. The effect of different doses of FSH *in vitro* on geometric mean (\pm s.e.m.) oestradiol production (pg/ml) between days 0-2 and between days 2-4 of culture (with day 0 being the first day of culture) from all follicles dissected from ovaries during seasonal anoestrus (Experiment 1).



5.3.1.4. Oestradiol production by growing follicles

There was no significant correlation between follicular size, either on day 0 or day 2 and geometric mean oestradiol production between days 0-2 and days 2-4 respectively. There was also no significant correlation between growth and geometric mean oestradiol production of follicles between days 0-2 or between days 2-4 of culture.

Within each treatment group, geometric mean oestradiol production by growing follicles for the first two days of culture did not differ from production during the last two days of culture (Figure 5.4.). Similarly, in non-growing follicles for each dose of FSH, there was no significant difference in geometric mean oestradiol production between days 0-2 and between days 2-4 of culture.

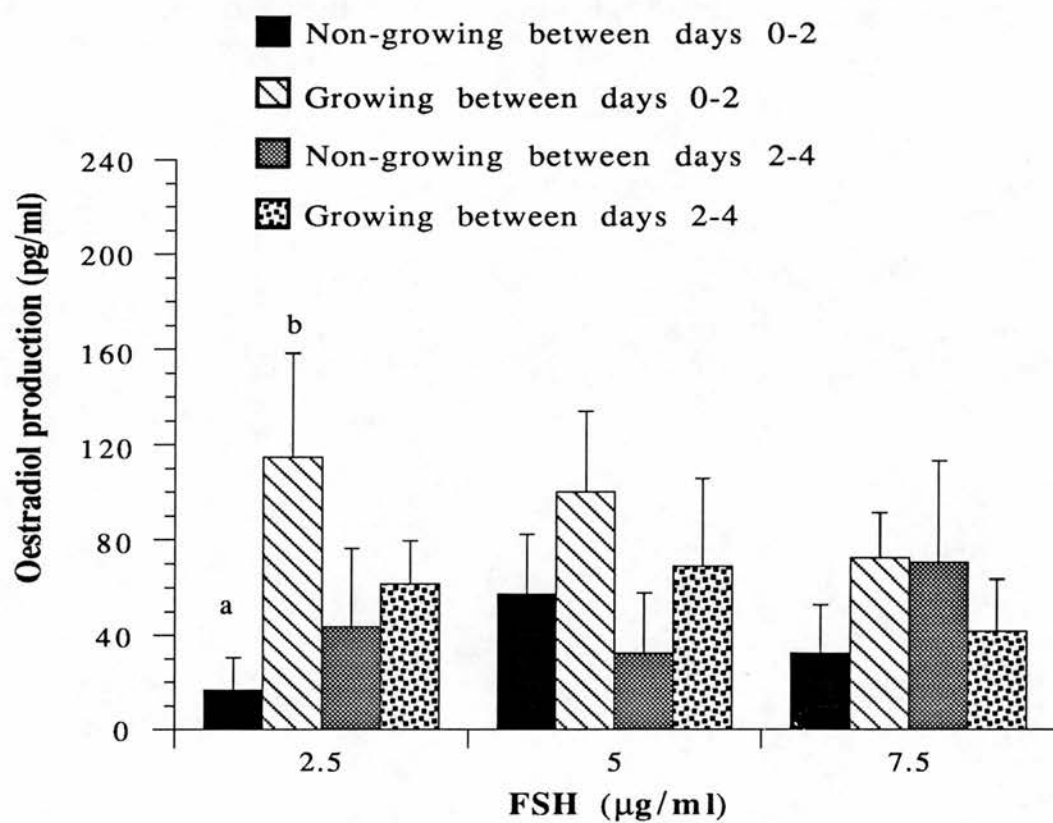
Geometric mean oestradiol production between days 0-2 was significantly greater ($P < 0.05$) in growing follicles incubated with the lowest dose of FSH ($2.5\mu\text{g/ml}$) compared to non-growing follicles in the same treatment group. However, geometric mean oestradiol production did not differ significantly between growing and non-growing follicles which had been incubated with either $5.0\mu\text{g/ml}$ or $7.5\mu\text{g/ml}$ FSH (Figure 5.4.).

5.3.2. Experiment 2

5.3.2.1. Size of all follicles

For all follicles, irrespective of the day of the luteal phase the follicles were explanted, there was no strong linear relationship between follicular size, either on day 0 or day 2 and the change in size between days 0-2 or between days 2-4 of culture.

Figure 5.4. The effect of different doses of FSH *in vitro* on geometric mean (\pm s.e.m.) oestradiol production (pg/ml) in culture media between days 0-2 and day 2-4 of culture (with day 0 being the first day of culture). Follicles were dissected from ovaries of Scottish Blackface ewes during seasonal anoestrus (Experiment 1). In the figure, the columns indicated by a and b differed significantly ($P < 0.05$).



On day 0, 1, 3 and 4 of culture, all follicles which were explanted on day 7 of the oestrous cycle had significantly ($P<0.05$) smaller mean diameters than follicles which were explanted on day 11 of the oestrous cycle (Figure 5.5.), with follicles explanted on day 9 being intermediate in size.

5.3.2.2. Size of growing follicles

There was no significant correlation between follicular size, either on day 0 or day 2 and growth between days 0-2 or between days 2-4 of culture. For growing follicles which were explanted on day 7, the daily mean increase in size (%) was not significantly different throughout the period of culture (Table 5.1.). In comparison, growing follicles which were explanted on day 9, increased significantly ($P<0.05$) in size (%) between days 2-3 compared to days 3-4 of culture. Also, growing follicles explanted on day 11 of the oestrous cycle, had a significantly greater ($P<0.05$) increase in size between days 0-1 compared to days 1-2, with values approaching significance ($P=0.053$) between days 3-4 of culture. In growing follicles explanted on day 9 of the oestrous cycle, mean increase in size (%) between days 0-1 of culture (Table 5.1.), was significantly smaller ($P<0.05$) than follicles which were explanted on either day 7 or day 11. Between days 1-2 of culture, growth of follicles dissected on day 11 of the oestrous cycle was significantly lower ($P<0.05$) than for follicles dissected on day 7. Between days 3-4 of culture, the increase in size of growing follicles explanted on day 9 of the oestrous cycle were significantly smaller ($P<0.05$) than follicles explanted on day 11.

Figure 5.5. The effect of stage of the oestrous cycle (days 7, 9 or 11) on the mean (\pm s.e.m.) diameter of all follicles cultured for 4 days with 2.5 μ g/ml FSH (Experiment 2). In the figure, the columns indicated by a versus b, c versus d differed significantly ($P < 0.01$) and e versus f ($P < 0.05$).

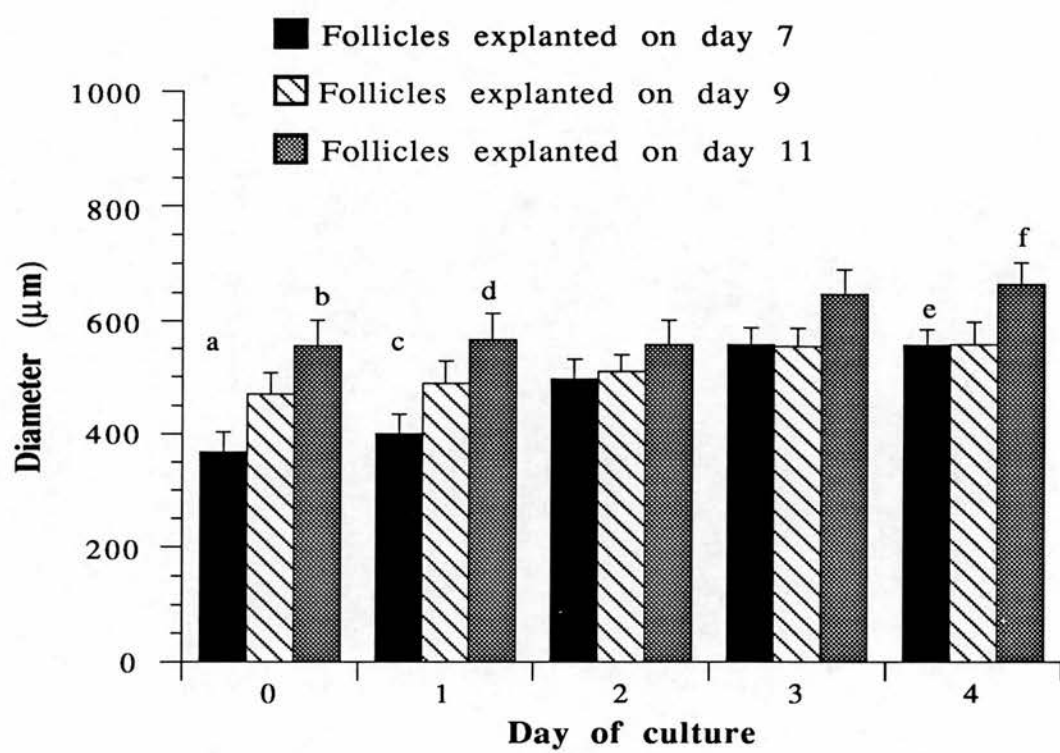


Table 5.1. Daily mean (\pm s.e.m.) increase in size (%) *in vitro* of growing follicles dissected from Scottish Blackface ovaries on different days of the oestrous cycle (Experiment 2).

Day of the oestrous cycle	Day of culture			
	0-1	1-2	2-3	3-4
7	13.0 \pm 2.3 g (n= 10)	17.7 \pm 3.8 f (n= 6)	10.9 \pm 2.8 (n= 5)	4.7 \pm 0.6 (n= 4)
9	6.0 \pm 1.5 h (n= 8)	12.4 \pm 3.1 (n= 7)	11.7 \pm 1.4 a (n= 8)	6.7 \pm 1.4 b (n= 5)
11	14.0 \pm 2.7 c (n= 2)	5.8 \pm 1.6 d (n= 4)	11.9 \pm 3.3 (n= 7)	35.1 \pm 19.1 e (n= 2)

Across rows a versus b, c versus d P<0.05

Down rows b versus e, c versus d, c versus h, f versus d, g versus h P<0.05

n = number of follicles

5.3.2.3. Oestradiol production by all follicles

There was no significant correlation between follicular size, either on day 0 or day 2 and geometric mean oestradiol production between days 0-2 or between days 2-4 of culture. For all follicles, there was no strong linear relationship ($r < 0.05$) between the mean change in the size of the follicle and geometric mean oestradiol production between days 0-2 or between days 2-4 of culture.

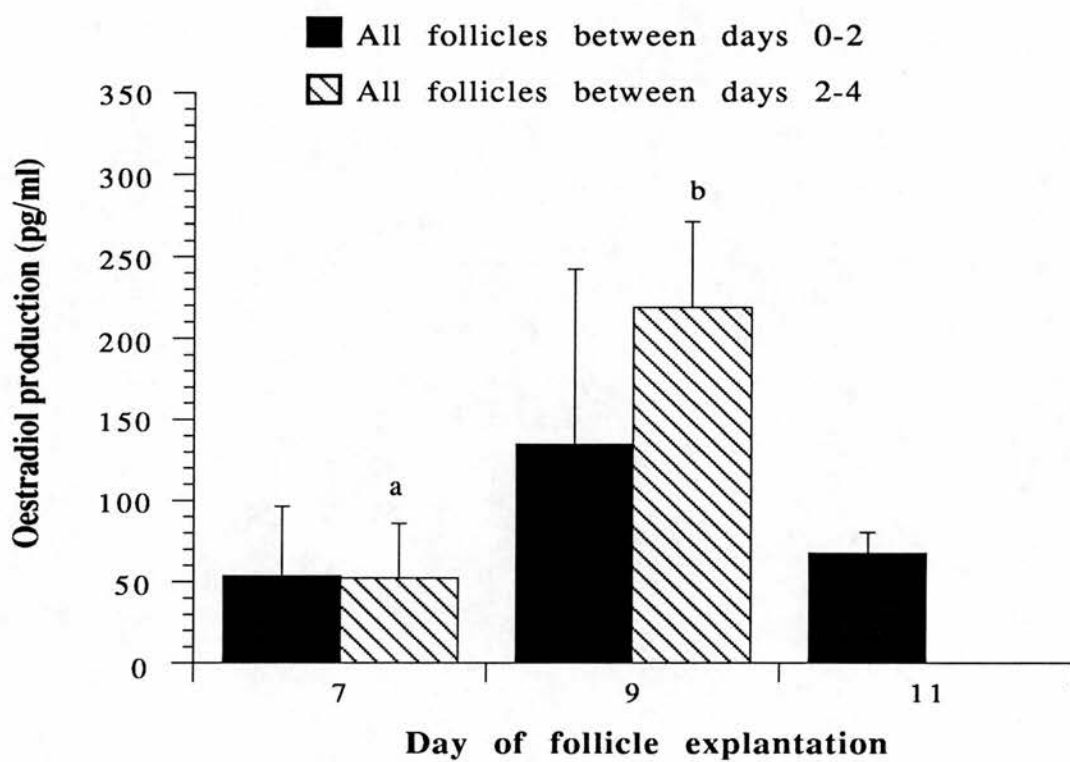
Irrespective of day of follicle explantation, there was no significant difference in geometric mean oestradiol production between days 0-2 of culture compared with days 2-4 (Figure 5.6.). Also, mean oestradiol production between days 0-2 did not differ between all follicles which were dissected on different days of the luteal phase. Geometric mean oestradiol production between days 2-4 of culture overall follicles dissected on day 9 of the oestrous cycle, was significantly higher ($P < 0.05$) compared to follicles explanted on day 7 (Figure 5.6.).

5.3.2.4. Oestradiol production by growing follicles

There was no significant correlation between follicular size, either on day 0 or day 2, and geometric mean oestradiol production between days 0-2 or between days 2-4 of culture respectively. There was also no significant correlation between follicular growth and geometric mean oestradiol production either between days 0-2, or between days 2-4 of culture.

For follicles explanted on day 7 of the oestrous cycle, comparison of oestradiol production between growing and non-growing follicles was not possible since all the follicles increased in size between days 0-2 of culture. In the day 11 group, growing and non-growing follicles did not differ

Figure 5.6. The effect of stage of the oestrous cycle (day 7, 9 or 11) on geometric mean (\pm s.e.m.) oestradiol production (pg/ml) by all follicles between days 0-2 and day 2-4 of culture, with day 0 being the first day of culture (Experiment 2). In the figure, the columns indicated by the letters a and b differed significantly ($P < 0.05$).



significantly in oestradiol production between days 0-2 of culture (**Figure 5.7.**). Geometric mean oestradiol production between days 0-2 of culture was significantly higher ($P<0.01$) in growing follicles compared with non-growing follicles which were explanted on day 9 of the oestrous cycle (**Figure 5.7.**). In addition, geometric mean oestradiol produced between days 0-2 by growing follicles explanted on day 9 was significantly greater ($P<0.05$ and $P<0.01$ respectively) than either the day 7 or day 11 groups. The media collected on day 4 of culture, from follicles explanted on day 11 of the oestrous cycle, was not retained for assay due to an excessive amount of cellular debris.

5.3.3. Experiment 3

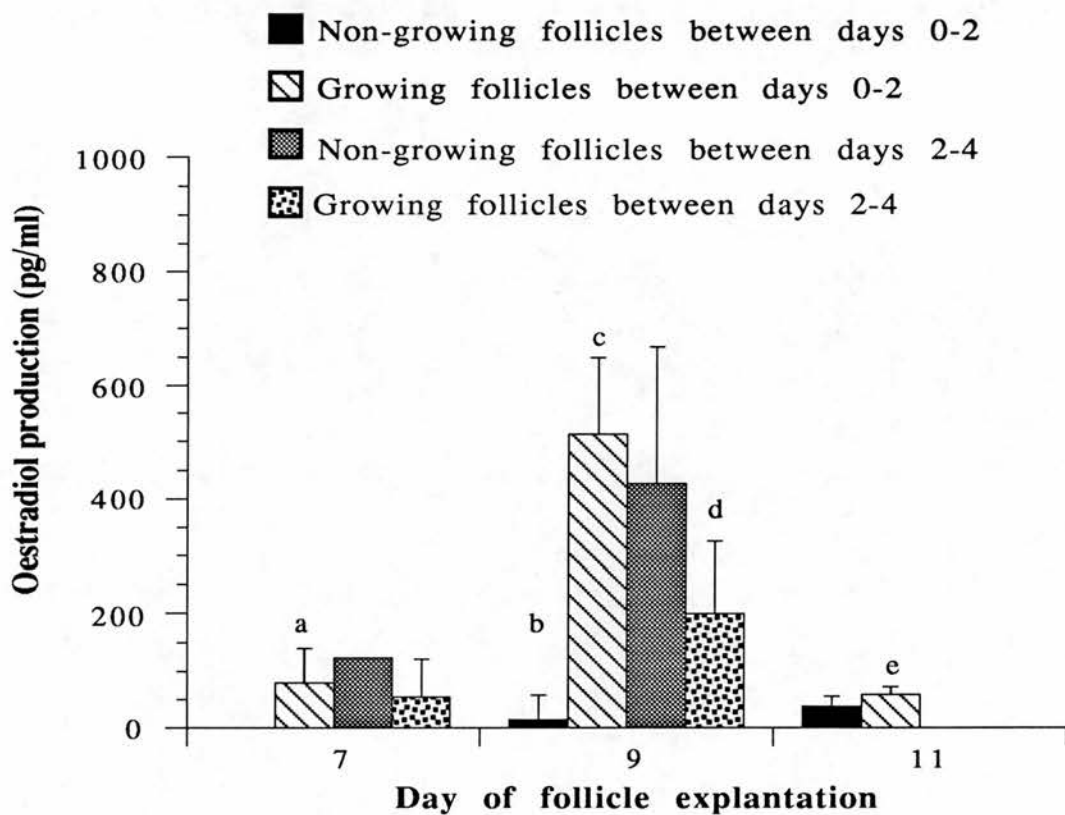
Follicles which were pre-incubated with ungassed α MEM, had a high, although temporary increase in pH which after 1 hour had risen to 8.1, this being outside the normal physiological range.

5.3.3.1. Size of all follicles

Mean size of all follicles on day 0 of culture was not significantly different between any of the treatment groups, that is, condition of pre-incubation media (**Table 5.2.**). Also, mean size of follicles did not differ significantly between desiccators (note: follicles from desiccator 1 were left undisturbed throughout the period of culture, whilst those from desiccator 2 were removed temporarily each day to allow for the measurement of follicular size).

Within desiccator 1, at the end of the culture period (day 3), there was no significant effect of using gassed or ungassed pre-incubation media on mean diameter of follicles obtained from homozygous carrier lambs (**Table 5.2.**). Within desiccator 2, for each genotype on any day of culture,

Figure 5.7. The effect of stage of the oestrous cycle (day 7, 9 or 11) on geometric mean (\pm s.e.m.) oestradiol production (pg/ml) by growing and non-growing follicles between days 0-2 and day 2-4 of culture, with day 0 being the first day of culture (Experiment 2). In the figure, the columns indicated by a versus b, b versus c differed significantly ($P<0.01$) and a versus c, c versus e ($P<0.05$).



mean diameter was not significantly different between follicles kept in ungassed pre-incubation media compared with gassed media (Table 5.2.).

For all follicles, there was a no significant correlation between size, either on day 0 or day 2 and the respective change in size between days 0-2 and between days 2-3. There was also no strong linear relationship ($r < 0.5$) between follicle size on day 0 and difference in size between day 0-3.

For all follicles obtained from homozygous Booroola Scottish Blackface lambs, which had been pre-incubated with gassed α MEM, daily removal (from desiccator 2) had no significant effect on growth compared to those which had been left undisturbed (desiccator 1) see Table 5.2. However, follicles obtained non-carriers and which had been pre-incubated with gassed α MEM were significantly larger ($P < 0.05$) when removed daily (desiccator 2) compared to those which had been left undisturbed (Table 5.2.). There was no significant genotypic difference in mean diameter of all follicles pre-incubated with ungassed media and then cultured either in desiccator 1 or 2. There was also no significant genotypic difference in mean diameter of all follicles pre-incubated with gassed media in desiccator 2. Follicles obtained from non-carriers and pre-incubated with gassed media, before being incubated in desiccator 2 were significantly smaller ($P < 0.05$) than follicles from homozygous lambs (Table 5.2.).

5.3.3.2. Oestradiol production by all follicles

There was no significant correlation between follicular size on day 0 and geometric mean oestradiol production between days 0-2 or between days 0-3 of culture. Similarly, there was no significant correlation between the change in follicular size and geometric mean oestradiol production between days 0-2, between days 2-3, or between days 0-3 of

Table 5.2. The effect of using gassed or ungassed pre-incubation media on the mean (\pm s.e.m.) diameter (μ m) of all follicles from Scottish Blackface ewe lambs possessing the Booroola Fecundity gene (Experiment 3).

Genotype	Desiccator	Preincubation media	DAY OF CULTURE			
			0	1	2	3
Fec ^B Fec ^B	1	α MEM	473 \pm 90 (n= 6)	----	----	468 \pm 73 (n= 5)
Fec ^B Fec ^B	1	α MEM + Gas	523 \pm 62 (n= 10)	----	----	564 \pm 50 a (n= 10)
Fec ^B Fec ^B	2	MEM	480 \pm 58 (n= 6)	495 \pm 64 (n= 6)	485 \pm 60 (n= 6)	490 \pm 64 (n= 6)
Fec ^B Fec ^B	2	α MEM + Gas	451 \pm 57 (n= 10)	513 \pm 66 (n= 8)	540 \pm 71 (n= 8)	594 \pm 59 (n= 7)
Fec ⁺ Fec ⁺	1	α MEM	481 \pm 38 (n= 10)	----	----	522 \pm 40 d (n= 9)
Fec ⁺ Fec ⁺	1	α MEM + Gas	350 \pm 35 (n= 4)	----	----	370 \pm 24 b (n= 4)
Fec ⁺ Fec ⁺	2	α MEM	483 \pm 46 (n= 10)	503 \pm 53 (n= 9)	510 \pm 58 (n= 8)	561 \pm 48 (n= 7)
Fec ⁺ Fec ⁺	2	α MEM + Gas	425 \pm 70 (n= 4)	480 \pm 38 (n= 4)	497 \pm 43 (n= 4)	512 \pm 46 c (n= 4)

Down rows a versus b, b versus c, b versus d P<0.05

n = number of follicles

culture. For both genotypes, daily removal of follicles, or gassing the pre-incubation media, did not significantly affect geometric mean oestradiol production (Table 5.3.).

5.4. Discussion

These studies show that small antral ovine follicles (200-800 μ m in diameter) can increase in size and produce oestradiol using an *in vitro* culture system modified from that used for mouse follicles by Nayudu and Osborn, unpublished observations; Nayudu *et al.* (1992).

Representative sections of haematoxylin and eosin stained follicles, which were either freshly dissected, or cultured for various lengths of time and then designated as 'growing' or 'non-growing', are shown in Figures 5.8 and 5.9 respectively. Growing follicles were symmetrical in shape, had well organised granulosa cell layers, an oocyte which was homogenous in appearance and prominent theca. Follicles which were designated as non-growing had certain common characteristics which included, the appearance of pycnotic bodies, irregular defined layers of granulosa cells and an oocyte which was granular in appearance.

At this stage of development, small ovine follicles (<1mm in diameter) were found to increase in diameter and produce small quantities of oestradiol irrespective of FSH concentration used in Experiment 1. This finding agrees with other authors whose evidence from *in vivo* studies suggest that basal folliculogenesis, for follicles less than 2mm in diameter (see section 1.4.1.), is partially independent of gonadotrophins (Driancourt and Fry, 1988; see review by Driancourt, 1991). In contrast, culture of large ovine follicles (>2mm in diameter) with FSH stimulates a significant increase in oestradiol production (Moor *et al.*, 1973). However, the

Table 5.3. The effect of using gassed or ungassed pre-incubation media on geometric mean oestradiol production (pg/ml; \pm s.e.m.) by follicles from either homozygous carrier or homozygous non-carrier lambs of the Booroola fecundity (Fec^B) gene (Experiment 3).

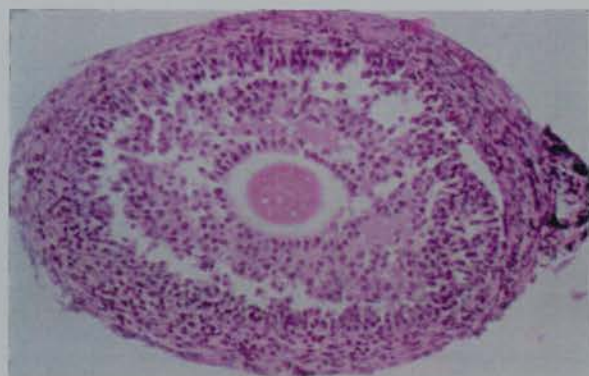
Genotype	Desiccator	Preincubation Media	Oestradiol production	
			Day 0-2	To day 3*
$Fec^B Fec^B$	1	α MEM	----	70.8 ± 59.4 (n= 8)
$Fec^B Fec^B$	1	α MEM + Gas	----	154.9 ± 104.1 (n= 12)
$Fec^B Fec^B$	2	α MEM	87.1 ± 73.1 (n= 8)	85.1 ± 67.6 (n= 8)
$Fec^B Fec^B$	2	α MEM + Gas	117.5 ± 58.2 (n= 12)	81.3 ± 40.3 (n= 12)
$Fec^+ Fec^+$	1	α MEM	----	72.4 ± 35.9 (n= 16)
$Fec^+ Fec^+$	1	α MEM + Gas	----	74.1 ± 17.7 (n=4)
$Fec^+ Fec^+$	2	α MEM	63.1 ± 23.6 (n= 12)	70.8 ± 9.9 (n= 11)
$Fec^+ Fec^+$	2	α MEM + Gas	120.2 ± 67.6 (n= 4)	112.2 ± 35.6 (n= 4)

* Note oestradiol production from follicles in desiccator 1 was measured between days 0-3, whilst oestradiol production by follicles in desiccator 2 was measured between days 2-3

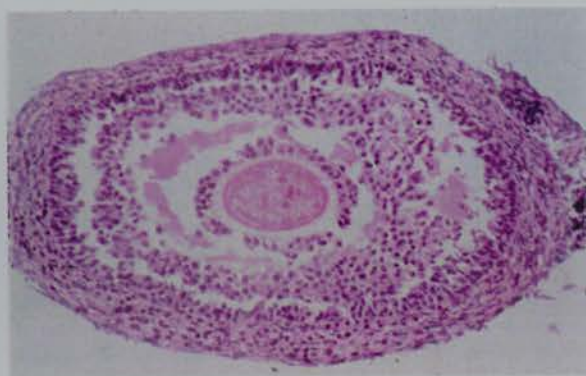
n= number of follicles

Figure 5.8. Panel (a) and (b) are representative haematoxylin and eosin-stained sections of freshly dissected follicles. The disassociation of the granulosa cell layers is thought to be caused by shrinkage caused by histological processing. Panels (c), (d), (e) and (f) are haematoxylin and eosin-stained sections of follicles which were cultured *in vitro* and classified as 'growing' between days 0-3. In all panels, follicles ranged from 310-610 μ m in diameter and were photographed at x100 magnification.

a



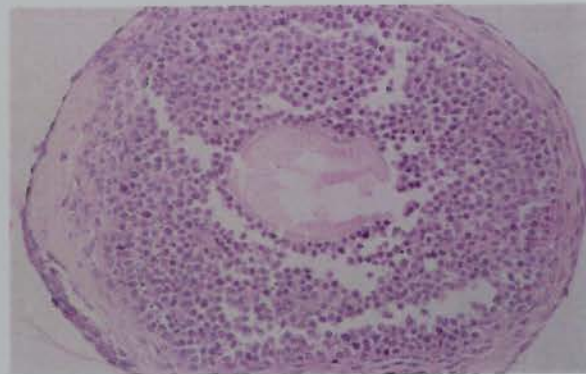
b



c



d



e



f

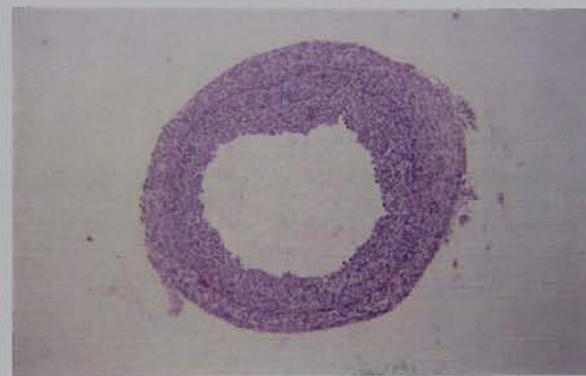
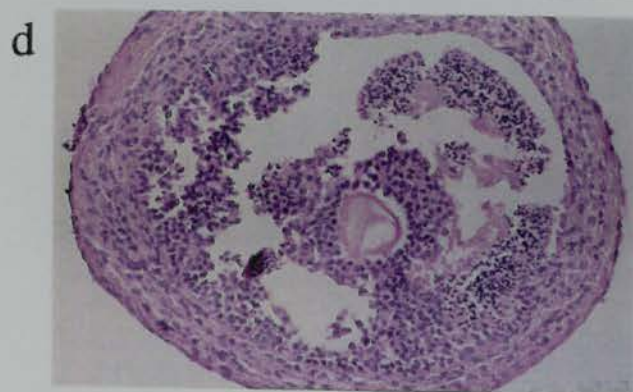
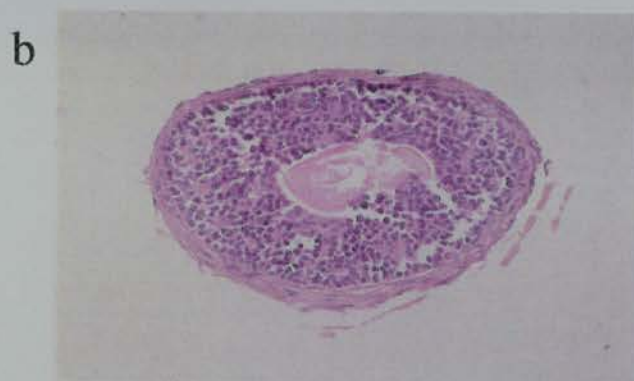


Figure 5.9. All panels are representative haematoxylin and eosin-stained sections of follicles which were cultured *in vitro* and classified as 'non-growing'. Follicles ranged from 410-910 μ m in diameter. The follicle shown in panel (a) was designated non-growing after 3 days while the other follicles shown are after 4 days in culture. All follicles were photographed at x100 magnification.



requirement for FSH *in vitro* for follicular growth and oestradiol production needs to be confirmed.

For any of the studies performed (Experiments 1-3), follicular size at the start of culture did not appear to be related to the change in size following several days. However, within each study, there was marked heterogeneity in the growth and oestradiol production from the follicle population. The reason for this marked heterogeneity between follicles in both growth and oestradiol production is not known, but may be due to either differences in follicular sensitivity, or response to either hormones and/or growth factors present in the serum. In addition, it cannot be excluded that there was a differential 'quality' between follicles, since not all follicles were examined histologically to determine their state of atresia. However, atresia rarely occurs in follicles < 1mm in diameter (Turnbull *et al.*, 1977), although premature atresia may have been induced by possible suboptimal culture conditions.

Interestingly, follicles which had been dissected on day 11 of the oestrous cycle were significantly larger when compared with those obtained on day 7 (Experiment 2). Despite this difference in size, mean oestradiol production did not differ significantly. An *in vitro* study of larger ovine follicles (2-4.5mm in diameter) also found no significant effect on oestradiol production by follicles explanted at different stages of the oestrous cycle (Moor, 1973). However, oestradiol production by follicles larger than 4.5mm in diameter was highly dependent on the day of the oestrous cycle (Moor, 1973; Seamark *et al.*, 1974). However, care must be exercised when comparing *in vitro* studies with different culture conditions.

McNatty *et al.* (1986) found that oestradiol production by follicles up to 0.53mm in diameter did increase with increasing diameter, although there was no effect of Booroola genotype. However, between 0.53 and 1mm in diameter oestradiol production remained largely unchanged. The physiological significance of this finding remains unclear and may well reflect the limitations of the culture system. It is also unclear whether the follicular diameter reported by these authors, related to the starting size of the follicle or the diameter after 48 hours of culture. In the three experiments reported in this study, there was no strong linear relationship between the increase in follicle size and geometric mean oestradiol production. The reason for this difference is not known but may be due to either differences in the culture conditions, or in the different breeds/strains of sheep.

In all three experiments, there were no significant correlation between follicle growth and geometric mean oestradiol production. This finding questions the importance of using oestradiol production as a marker to assess the maturity of follicles at this particular stage of development. Furthermore, small follicles have a limited capacity to produce oestradiol, probably due to a limited number of LH receptors on the granulosa cells (see section 1.6.3.). However, these studies did not investigate the role of LH upon growth and steroidogenesis of ovine follicles *in vitro*. This may be a useful avenue for future work since *in vivo* evidence suggests that administration of FSH and LH to increase ovulation rate is thought to be dependent on the proportion, or ratio, of FSH to LH (Cognié *et al.*, 1986; Wright *et al.*, 1991)

The fact that fewer follicles were increasing in size towards the end of the culture period suggests that the conditions employed were

suboptimal and therefore unable to maintain follicular growth through to the final stages of development. This inability of the majority of ovine follicles to survive long-term culture highlights important species differences, since in mice, using similar culture techniques, preantral follicles can be grown to the large antral stage (Nayudu *et al.*, 1992). Interestingly, both studies cultured follicles for similar time periods, although the rate of growth for mouse follicles was greater than for ovine follicles, highlighting species differences. The failure of ovine follicles to be maintained under long-term culture conditions may be due to a number of factors including a lack of a specific metabolic substrate and/or an insufficient stimulus from a required growth factor. Further possible complications may be due to failure of substrates and/or oxygen to diffuse into the follicle because of its size. In addition, follicles may also be adversely affected by the length of time required for, and the difficulty in, manually dissecting them from sheep ovaries. However, temporarily placing follicles, from either genotype, into ungassed α MEM pre-incubation media had no significant adverse effects, either on the change in size, or oestradiol production of all follicles. In view of the observed rise in pH that occurs with ungassed α MEM, it was decided that for future experiments pre-incubation of follicles would only be carried out with gassed media. The daily, although temporary, removal of follicles from the incubator did not adversely affect either oestradiol production or percentage increase in size of the follicles from either genotype. This is important since in future experiments the development (size and oestradiol production) of follicles needs to be monitored frequently due to the heterogeneity in the follicle population.

Booroola fecundity gene specific differences in lambs have been demonstrated by; earlier maturation of lambs despite similar liveweights

(Bindon *et al.*, 1982); the number of primordial follicles in new-born lambs (Tassell, Kennedy, Bindon and Piper, 1983); plasma pituitary hormone concentrations (Bindon and Turner, 1974; Findlay and Bindon 1975; Bindon, Findlay and Piper, 1985) and pituitary response to gonadotrophin-releasing hormone (Bindon 1984; McNatty and Henderson, 1987; Braw-Tel and Gootwine, 1989). It is interesting to note that, in this preliminary study, no gene specific differences were found in mean oestradiol production from small follicles derived from Booroola Scottish Blackface lambs.

In summary, a method for the manual dissection and culture of small ovine follicles has been developed. This simple approach can be used to investigate factors involved in the control of follicular development. Preliminary evidence from this study suggests there are no genotypic differences in oestradiol production by follicles obtained from lambs which were either homozygous carriers or non-carriers of the Booroola fecundity gene. However, because of the marked heterogeneity within the follicle populations larger numbers of follicles are required to confirm this result. In conclusion, further work is required involving a larger number of animals, to test the hypothesis that gene specific differences exist in follicular responsiveness to FSH, in terms of growth and oestradiol production.

CHAPTER SIX

The effect of FSH *in vitro* on follicular growth and steroidogenesis

6.1. Introduction

Previous *in vivo* results show that genotypic differences in peripheral FSH are not wholly responsible for controlling the genotypic differences in ovulation rate within our defined populations of Booroola Scottish Blackface ewes (Chapter 3 and 4). As well as differences in peripheral FSH, there may be genotypic differences in follicular responsiveness and/or sensitivity to FSH. Follicular response to FSH, in terms of growth and/or steroidogenesis, will depend not only on peripheral concentrations of FSH at particular stages of development, but also on the ability of the follicle to respond to the fluctuating FSH levels.

The development of an *in vitro* monolayer culture system for one or more of the follicular cell types, provides a useful experimental model to study the role of hormones and/or growth factors in follicular growth and steroidogenesis. However, an *in vitro* whole follicle culture system (Chapter 5), compared to monolayer culture, has the distinct advantage of maintaining the ultrastructural relationships between theca, granulosa cells and oocyte, thus allowing the possibility of paracrine and/or autocrine mechanisms to operate.

The aim of this study was to test the hypothesis that there are genotypic differences in the pattern of follicular development which are due to differences in follicular responsiveness (growth or oestradiol production) to FSH, within two populations of Booroola Scottish Blackface ewes.

6.2. Materials and Methods

6.2.1. Animals

The animals used in this study were either from the backcross or F₂ population. The animals used in Experiment 1 were backcross Booroola Scottish Blackface ewes, 2-4 years of age and were classified by pedigree analysis as being either Fec^BFec⁺ (n=6) or Fec⁺Fec⁺ genotype (n=6) on the basis of at least 5 previous ovulation rate records. The animals used in Experiment 2 were F₂ Booroola Scottish Blackface ewes, 2-4 years of age and were also classified based on pedigree analysis as being either Fec^BFec^B (n=10), Fec^BFec⁺ (n=8) and Fec⁺Fec⁺ genotype (n=8) as described in Chapter 4.

6.2.2. Experimental protocol

6.2.2.1. Effect of FSH on growth and oestradiol production by follicles from the backcross population (Experiment 1)

During seasonal anoestrus (July), two ewes (one of each genotype) were ovariectomized each day as described in section 2.2.3. The genotypes of the animals was not decoded until the end of the period of culture work. The absence of a corpus luteum was evidence that ewes were anoestrus. The procedure for the culture of the follicles was as described in section 2.5. whereby follicles were cultured for 4 days with the media being changed and collected on day 2 and collected on day 4. The culture media had the same components as described in section 5.2.2.3., except that ewe lamb serum obtained from our own flock was used in place of the pooled lamb serum obtained from Life Technologies, where the pooled lamb serum originated from lambs of unknown age or sex. The dose of FSH supplemented in the culture media was 2.0µg/ml (300ng/well).

6.2.2.2. Effect of FSH on growth and oestradiol production by follicles from the F₂ population (Experiment 2)

During the breeding season (December) F₂ Booroola Scottish Blackface ewes were synchronised using vaginal progestagen sponges for 12 days (section 4.2.2.2.). Briefly, all ewes were run with a raddled ram and checked twice daily for oestrus, with day 0 being the day of behavioural oestrus. On day 8 of the oestrous cycle, all ewes were ovariectomized and follicles dissected and incubated as described previously (section 5.2.2.3.), except that the lamb serum used was from our own flock as described above. The culture media was supplemented with FSH, either 0.5µg/ml (75ng/well) or 2.0µg/ml (300ng/well).

6.2.3. Hormone assay

6.2.3.1. Oestradiol

The concentrations of oestradiol in culture media, holding media and dissection media were determined using a non-extraction radioimmunoassay as described in section 2.3.3. The minimum detectable value was 27.0pg/ml. The inter-assay and the intra-assay CV were 5.5% and 8.3% respectively.

6.2.3.2. Statistical analyses

Data which were skewed in distribution, as assessed by the Statworks package on the Apple Macintosh computer, were logarithmically transformed before being expressed as geometric means (\pm s.e.m.). Normally distributed data are expressed as arithmetic means (\pm s.e.m.). Data were compared either by Student's *t* test, Chi-square analysis (χ^2) or by analysis of covariance using the Minitab programme on the Apple

Macintosh computer. A covariance model was used to allow multiple comparisons of the data. Size, growth, treatment and genotype variables used in the covariance model accounted for <30% of the variation of any of the dependent variables investigated (oestradiol production, growth or size).

6.3. Results

6.3.1. Experiment 1

6.3.1.1. Follicle size

For all follicles, there was no strong linear relationship (r was <0.5) between size, either on day 0 or day 2, and change in size between days 0-2 or between days 2-4 of culture respectively. FSH had no significant effect on the daily mean size of all follicles within each genotype. Also, there was no significant genotypic difference in mean diameter of all follicles in response to FSH and so values from the different FSH treatment groups were pooled, within a genotype, for further analyses. Mean diameter for all follicles, irrespective of dose of FSH, did not differ between genotypes on day 0, but follicles from non-carrier ewes were significantly larger compared with follicles obtained from heterozygous ewes on days 1, 3 and 4 of culture (Table 6.1.).

6.3.1.2. Follicle growth

Follicles were classified as 'growing' if the diameter increased beyond the limit of detection of the graticule, $10\mu\text{m}$ (see section 5.1.). Follicular size, either on day 0 or day 2, was not significantly correlated with growth between days 0-2 or between days 2-4 of culture, indicating that larger follicles do not necessarily grow more than smaller follicles. For follicles obtained from both heterozygous and non-carrier ewes, FSH had a significant ($P<0.001$ and $P<0.05$ respectively) effect upon follicular

Table 6.1. Mean (\pm s.e.m.) diameter (μ m) of all follicles, irrespective of dose of FSH, from the backcross population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 1).

Genotype	Day of Culture				
	0	1	2	3	4
$Fec^B Fec^+$	489 \pm 19 (n=46)	477 \pm 21 ^a (n=36)	499 \pm 22 (n=37)	505 \pm 24 ^a (n=29)	491 \pm 25 ^a (n=21)
$Fec^+ Fec^+$	530 \pm 19 (n=70)	535 \pm 18 ^b (n=66)	555 \pm 18 (n=66)	573 \pm 19 ^b (n=55)	588 \pm 25 ^b (n=41)

Down rows a versus b, $P<0.05$

growth between days 0-2, but not between days 2-4 of culture, although the number of follicles in this group was small (Table 6.2.). There was no significant genotypic effect of FSH on follicular growth between days 0-2 or between days 2-4 of culture.

6.3.1.3. Oestradiol production by growing follicles

For heterozygous carriers of the Booroola fecundity gene, FSH significantly ($P<0.05$) increased geometric mean oestradiol production by growing follicles between days 0-2 of culture (Table 6.3.). In contrast, FSH had no significant effect on mean oestradiol production between days 0-2 by growing follicles from non-carriers of the Fec^B gene. Also, mean oestradiol production for growing follicles cultured with FSH between days 0-2 did not differ significantly ($P=0.07$) between genotypes (Table 6.3.).

For each genotype, there was no significant effect of FSH on geometric mean oestradiol production by growing or non-growing follicles between days 2-4 of culture (Table 6.3.). Similarly, there were no genotypic differences in the effect of FSH on oestradiol production by growing follicles, between days 2-4 of culture. Geometric mean oestradiol production by non-growing follicles cultured without FSH between days 2-4 of culture was significantly ($P<0.05$) greater in non-carrier ewes than heterozygous ewes (Table 6.3.).

6.3.2. Experiment 2

6.3.2.1. Follicle size

For each genotype, there was no consistent effect of FSH on mean diameter of all follicles and so values were pooled to allow further analyses (Table 6.4.). On all days, mean size of all follicles obtained from homozygous carriers of the Booroola fecundity gene were always

Table 6.2. The effect of FSH on the mean (\pm s.e.m.) growth (μ m) between days 0-2 and days 2-4 of culture by follicles from the backcross population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 1).

Genotype	FSH Dose (μ g/ml)	Day of Culture	
		0-2	2-4
$Fec^B Fec^+$	0	32.2 ± 4.6^a (n=9)	30.0 (n=1)
$Fec^B Fec^+$	2.0	90.0 ± 15.1^b (n=7)	40.0 ± 0.0 (n=2)
$Fec^+ Fec^+$	0	42.0 ± 6.3^c (n=10)	30.0 ± 7.8 (n=5)
$Fec^+ Fec^+$	2.0	75.6 ± 10.9^d (n=18)	53.8 ± 9.3 (n=8)

Down rows a versus b, $P < 0.001$, c versus d $P < 0.05$

n = number of follicles

Table 6.3. Geometric mean (\pm s.e.m.) oestradiol production (pg/ml) by growing and non-growing follicles, between days 0-2 and days 2-4 of culture, from the backcross population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 1).

Days of Culture	Follicle Status	Fec^B	Fec^+	Fec^+	Fec^+
		Control	FSH	Control	FSH
0-2	Growing	55.0 ± 11.7^a (n=9)	186.2 ± 111.3^b (n=7)	72.4 ± 46.0 (n=10)	56.2 ± 19.4 (n=18)
0-2	Non-Growing	89.1 ± 25.9 (n=11)	93.3 ± 43.3 (n=8)	57.5 ± 16.7 (n=21)	55.0 ± 19.0 (n=14)
2-4	Growing	66.3 (n=1)	33.9 ± 0.2 (n=2)	123.0 ± 78.0 (n=5)	89.1 ± 35.9 (n=8)
2-4	Non-Growing	45.7 ± 7.5^c (n=10)	69.2 ± 18.3 (n=7)	89.1 ± 16.8^d (n=13)	58.9 ± 22.0 (n=15)

Across rows a versus b, c versus d $P < 0.05$

n= number of follicles

significantly smaller (Table 6.4.) than follicles obtained from non-carrier ewes. Generally, follicles obtained from heterozygous ewes were of an intermediate size, compared to follicles obtained from homozygous or non-carrier ewes, and were only significantly larger ($P < 0.05$) than follicles from homozygous ewes on day 1 of culture (Table 6.4.).

6.3.2.2. Follicle growth

A follicle was classified as 'growing' if the diameter increased beyond $10\mu\text{m}$ (see section 5.1.). Between genotypes, there was no consistent significant effect of FSH either on the increase in diameter or the proportion of follicles which grew, between days 0-2 or days 2-3 of culture, so values were pooled for further analyses. Mean growth of follicles, irrespective of genotype, or dose of FSH, was $63 \pm 4\mu\text{m}$ ($n=146$) and $46 \pm 4\mu\text{m}$ ($n=70$) between days 0-2 and days 2-3 respectively. FSH significantly increased the proportion of follicles which grew between days 0-2 and days 2-3 of culture (Table 6.5.).

6.3.2.3. Oestradiol production by growing follicles

Within each genotype, increasing doses of FSH had no significant effect on geometric mean oestradiol produced by growing follicles between days 0-2 and between days 2-3. Furthermore, there was no significant genotypic effect on geometric mean oestradiol production by growing follicles between days 0-2 or between days 2-3 of culture. In view of the lack of significant differences between the genotypes, or in the dose of FSH used, values were pooled for further analyses. FSH did not significantly affect the proportion of growing follicles producing oestradiol (approximately 80%) between days 0-2 or between days 2-3. Geometric mean oestradiol production was not significantly affected by growth. However, the larger the size of the growing follicle, either on day 0 or day

Table 6.4. Mean (\pm s.e.m.) diameter (μ m) of all follicles, irrespective of treatment group, which were dissected on day 8 of the oestrous cycle from the F₂ population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 2).

Genotype	Day of Culture			
	0	1	2	3
Fec ^B Fec ^B	344 \pm 19 ^a (n=58)	369 \pm 17 ^c (n=72)	393 \pm 16 ^f (n=77)	408 \pm 20 ^h (n=53)
Fec ^B Fec ⁺	394 \pm 23 ^{a,b} (n=54)	425 \pm 20 ^c (n=80)	440 \pm 20 ^{f,g} (n=82)	444 \pm 24 ^{h,i} (n=63)
Fec ⁺ Fec ⁺	415 \pm 15 ^b (n=74)	429 \pm 12 ^d (n=100)	444 \pm 13 ^g (n=97)	461 \pm 13 ^j (n=85)

Between genotypes a versus b, c versus d $P < 0.01$; c versus e, f versus g, h versus j $P < 0.05$

Table 6.5. The effect of FSH on the proportion (%) of growing or non-growing follicles between days 0-2 and between days 2-3 of culture. The follicles were explanted on day 8 of the oestrous cycle from the F₂ population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 2).

FSH dose (μ g/ml)	Follicle status			
	Days 0-2		Days 2-3	
	Non-Growing	Growing	Non-Growing	Growing
0	56 (n=47)	44 (n=37)	80 (n=53)	20 (n=13)
0.5	40 (n=33)	60 (n=49)	60 (n=42)	40 (n=28)
2.0	30 (n=26)	70 (n=60)	58 (n=40)	42 (n=29)

n = number of follicles

Increasing dose of FSH significantly increased the proportion of follicles growing between days 0-2 ($X^2 = 11.1$, $P < 0.01$) and between days 2-3 ($X^2 = 9.1$, $P < 0.05$) of culture

2, the greater the geometric mean oestradiol production ($P < 0.01$ between days 0-2 and $P < 0.05$ between days 2-3 respectively). For each genotype, over the initial 48 hour culture period, geometric mean oestradiol production was not significantly different from that produced in the subsequent 24 hour period (Table 6.6.).

6.4. Discussion

These data show that, in response to FSH, there were no genotypic differences in the growth or oestradiol production of small follicles ranging from 0.2-0.8mm in diameter which had been obtained from either the backcross, or the F_2 population.

Although size was not a useful predictor of subsequent growth for any of the follicle populations, in agreement with the results in Chapter 5, it appeared to be significantly related to the ability to produce oestradiol. The lack of a significant correlation between growth and oestradiol production suggests that oestradiol may be a poor marker in assessing the potential of these small follicles to develop. It is known that, for this stage of development, follicular fluid has a high concentration of testosterone and relatively low levels of oestradiol (Moor *et al.*, 1978; England *et al.*, 1981; Armstrong *et al.*, 1981), hence in retrospect testosterone production may have been a more useful marker to assess maturity and should be investigated further. Unfortunately, testosterone could not be measured in this study as the remaining sample volume was too small. In contrast to the high androgen concentration in small follicles, where rates of atresia are virtually zero (Turnbull *et al.*, 1977), atresia is often associated with high concentrations of androgen in larger follicles (Moor *et al.*, 1978; Carson *et al.*, 1979 and Tsafiriri and Braw, 1984), however the difference between these two states remains an enigma (Hunter *et al.*, 1992).

Table 6.6. Geometric mean oestradiol production between days 0-2 and days 2-3 of culture by growing and non-growing follicles which were producing oestradiol and were obtained from the F₂ population of Scottish Blackface ewes with or without the Booroola fecundity (Fec^B) gene (Experiment 2).

Follicle Status	Oestradiol Production (pg/ml)	
	Days 0-2	Days 2-3
Non-growing	93.3 ± 13.1 (n=86)	72.4 ± 8.4 (n=103)
Growing	96.0 ± 11.1 (n=115)	89.1 ± 12.5 (n=53)

n = number of follicles

In this study, the marked heterogeneity in growth and oestradiol production for similar-sized follicles may be due to either a difference in response to FSH, or as a result of different biosynthetic capabilities between follicles. Interestingly, despite these differences in growth and steroidogenic response of similar sized follicles to FSH *in vitro*, rates of atresia *in vivo* are virtually zero (Turnbull *et al.*, 1977). This suggests that a low response to FSH has no detrimental effect on the survival of the follicle at this stage of development. It is unknown whether, at this stage of development, the follicles which produce more oestradiol and/or grow more than their counterparts are the ones which are preferentially chosen at recruitment. The possibility that the marked heterogeneity in growth and oestradiol production of similar sized follicles is induced by suboptimal culture conditions cannot be excluded, since the numbers of growing follicles declined towards the end of culture in both experiments. However, those follicles which continued to grow and produce oestradiol towards the end of culture, between days 2-3, did so at a faster rate than those follicles cultured between days 0-2 (Experiment 1). This finding was unexpected since the results from Chapter 5 showed that follicular growth and oestradiol production were not significantly different between days 0-2 and days 2-4 of culture. However, it is not known whether the follicles discussed in the previous experiments (Chapter 5) also had an increase in growth and oestradiol production between days 2-3, followed by a decline between days 3-4, such that mean growth and oestradiol production between days 2-4 was similar to those follicles cultured between days 0-2 of culture.

McNatty *et al.* (1986a) incubated follicles <1mm in diameter with FSH (1µg/ml) for 48 hours and found no genotypic difference in the amount of oestradiol produced, similar to the findings for this work. Care must be exercised however, when comparing *in vitro* studies, since differences

exist in the use of culture methodologies (dose of FSH or culture media), breed/strains or age of ewes and stage of the oestrous cycle, which all limit the validity of direct comparison. These above factors may help to explain why McNatty *et al.* (1986a) found genotypic differences ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}} > \text{Fec}^{\text{B}}\text{Fec}^{+} > \text{Fec}^{+}\text{Fec}^{+}$) in the proportions of follicles producing oestradiol, while we found no such genotypic differences.

Carriers of the Booroola fecundity gene have preovulatory follicles which are smaller in diameter compared to non-carriers (Scaramuzzi *et al.*, 1983), in common with other prolific breeds (see section 1.4.3.). Interestingly, in our study, the overall mean diameter of follicles obtained from homozygous ewes was significantly smaller compared with those from non-carriers within the F_2 population (Table 6.4.). The overall mean diameter between follicles obtained from heterozygotes compared to non-carriers in either the backcross or F_2 population was not significantly different. This genotypic difference in the overall mean follicle diameters is difficult to explain, but may be an indication that the Booroola fecundity gene exerts an effect from the earliest stages of development, although the proportions of small antral follicles are thought not to differ between genotypes (Driancourt *et al.*, 1985).

FSH was found to increase the growth of follicles in Experiment 1 and yet only increased the proportion of follicles which grew in Experiment 2. It seems unlikely that there is a biological difference between the follicles from the two populations in response to FSH and the results may be due to the effect of season, or the change in the source of lamb serum, which may have had a different milieu of hormones and/growth factors. Despite the difference in the effect of FSH in the two experiments, there was no genotypic difference in response to FSH. Further evidence that there are no genotypic differences in follicular

response to FSH come from the lack of differences in FSH binding characteristics to the component tissues of the follicle (McNatty *et al.*, 1989) and investigations into the biosynthetic capability of the follicle in terms of mean peak cAMP production (Henderson *et al.*, 1985; Henderson, McNatty, O'Keefe, Lun, Heath and Prisk, 1987; McNatty *et al.*, 1990).

The possibility that genotypic differences exist in response to FSH towards the latter stages of follicular development cannot be excluded, since ewes possessing the Booroola fecundity gene are thought to have an extended recruitment phase and lower intensity of selection (Driancourt *et al.*, 1985). However, the exact mechanisms controlling the recruitment and selection of ovine follicles are not fully understood (section 1.4.). This study concentrated on the possible genotypic differences in follicular responsiveness to FSH, although differences in follicular sensitivity to FSH may be more important, particularly towards the latter stages of development when follicles from carriers of the Booroola fecundity gene are thought to 'resist' atresia in order to 'wait' for ovulation (Driancourt *et al.*, 1985). The factors which are involved in the mechanisms of atresia are not fully understood, although they may well involve the action of growth factors. The intra-follicular concentration of oestradiol and IGF-1 may enhance the effects of FSH, thereby contributing to the establishment of a follicular threshold to FSH (see Hunter *et al.*, 1992 for review). This may be important in determining whether the follicle ovulates or becomes atretic. EGF(TGF α)-binding sites have been located on the theca in healthy ovine follicles, although the number and distribution of these decrease in the theca and increase in the granulosa layer as the follicle becomes atretic (Murray, Mariana and Driancourt, 1991). Clearly, further study is required to resolve the role of growth factors in follicular growth and development. The use of an *in vitro* system to culture whole follicles could prove to be a

useful, although relatively short-term, model to study the effects of growth factors and their interaction with hormones. The main disadvantage of this culture system is the reduced numbers of follicles which continue to grow beyond 3 days. The relatively short lifespan of these follicles not only limits the length of time they can be cultured, but also questions how biologically relevant their growth and development is *in vitro* compared with *in vivo* studies.

In conclusion, there were no genotypic differences in growth or oestradiol production in response to FSH by small antral follicles from either the backcross, or the F₂ population of Booroola Scottish Blackface ewes.

CHAPTER 7

***In vivo* studies of possible genotypic differences in follicular growth using the ovariectomized severe combined immunodeficient (SCID) mouse model**

7.1. Introduction

Previous attempts by this author to study growth and steroidogenesis of small ovine follicles *in vitro* (Chapter 6) has had limited success, due to an inability to maintain the follicles for more than a few days. This model, whilst advantageous in allowing reasonable short-term investigations into factors controlling follicular growth and steroidogenesis, does not enable a more long-term approach to be employed. The lack of an appropriate system, or model, to investigate the effect of hormones and/or growth factors upon follicle growth and development limits investigations into possible genotypic differences in follicular responsiveness (growth or steroidogenesis) to FSH for ewes possessing the Booroola fecundity gene.

The transplantation of ovaries is a well-established technique in experimental biology and has been used to investigate follicular growth and development (Krohn, 1977). The technique of transplantation using allografts (grafting between donors and hosts of the same species) has been widely used for mice and rats, but presents a potential rejection problem for farm animals, since ovaries are not immunologically privileged and farm livestock are not highly inbred like laboratory strains of mice (see Gosden, 1992a for review). The use of xenografts (grafting between donors and recipients of different species) would therefore be more beneficial for domesticated animals, although preferably dependent upon the host being immunologically deficient.

The work described in this Chapter examines the potential of a new xenograft technique which has been applied for the first time to domestic livestock. This technique involved the use of severe combined immunodeficiency (SCID) homozygous mice whose genetic background is Balb/C. SCID mice have a congenital defect that impairs both T and B lymphocyte immunity (Bosma, Custer and Bosma, 1983; Custer, Bosma and Bosma, 1985; Bosma, Davisson, Ruetsch, Sweet, Shultz and Bosma, 1989) and consequently they can be used as hosts in xenograft transplantations. The donor material was of ovarian origin and obtained from Booroola Scottish Blackface sheep, whose genotypes are known to differ in their pattern of follicular development (see section 1.5.). Our studies have shown that genotypic differences in peripheral FSH concentrations fail to explain fully the genotypic differences observed in mean ovulation rates. In addition, there was no genotypic difference in the responsiveness of small follicles to FSH *in vitro*. Taken together these data question the role of FSH in the observed genotypic differences in follicular development in Booroola Scottish Blackface ewes and lead to speculation about mechanisms of ovarian control.

The aim of this experiment was to attempt to establish the xenografting technique, specifically by using ovariectomized SCID mice as hosts and donor ovarian material from Booroola Scottish Blackface sheep. The work investigates the potential of this new technique as a tool to examine whether the genotypic differences in follicular development are still present *in vivo* when follicles are exposed to the same gonadotrophic environment.

7.2. Materials and Methods

7.2.1. Animals

The lambs used in this experiment were 6-12 months of age and had been assigned to either the $Fec^B Fec^B$ or $Fec^+ Fec^+$ genotype as described in Chapter 5.

Immunologically deficient SCID mice were kept in a positive pressure isolator (Moredun, Scotland) and received a weekly dose (half a tablet per 300ml double distilled autoclaved water) of the antibiotic Septrin (Wellcome, England). The animals ($n=9$) were fed *ad libitum* with irradiated 0.025Gray CRM SDS diet. The animals used in this study were females of 8 weeks of age and weighed approximately 30g.

7.2.2. Experimental protocol

The lambs were ovariectomized as described in section 2.5. and the ovaries transferred aseptically into Leibovitz-L15 culture media. The mice were anaesthetized by Avertin inhalation and then dorsally shaved (30-40mm) before the exposed skin was sprayed with a topical antiseptic. The host ovaries were aseptically removed after a dorso-lateral incision, since it is known from autografting experiments that grafts develop more satisfactorily in ovariectomized animals (Boot 1955; Ingram and Krohn, 1956). The improved development of grafts in ovariectomized animals is probably as a result of an increase in circulating levels of gonadotrophins, which stimulate a higher level of regeneration and growth of the graft (Harris and Eakin, 1949). Several sections (approximately 1mm^3) of ovarian (non-luteal) cortical tissue were removed from the ovine ovary using a scalpel blade. The gross morphology of the cortical tissue showed no large antral follicles present. The ovarian sections were placed under

the left renal capsule of the mouse, with the outer surface of the ovarian tissue being placed uppermost under the kidney capsule. Ovarian material from lambs was used in this experiment because it contains a higher number of primordial follicles and the cortex is less fibrous, thus favouring rapid revascularization compared to the cortex from adult ewes (Gosden, 1992b).

The kidney is highly vascularized and should allow better cell survival than other organs or tissues (Gosden, 1992b), whilst the renal membrane also provides a convenient pocket for anchorage. The left kidney capsule was used preferentially as it is positioned more caudally than the right kidney capsule within the body cavity and thus provides easier surgical access. In all cases, only one recipient kidney was involved in the grafting experiments, since in the unforeseen event of subsequent failure of that kidney to function correctly, the animal would still survive. The dorso-lateral incision of the body wall was closed with suture and several Michel clips. After surgery, the animals were kept warm and allowed to recover before being replaced into the incubator.

For all mice, the vagina closed within three days post-surgery. Initially, the ovariectomized animals were monitored daily for signs of vaginal opening, but when this had not occurred within several months the animals were then monitored twice weekly. The vagina was graded as 'closed', 'slightly open' or 'open' as a biological-indicator for oestrogenization. If the animal exhibited persistent vaginal opening, or had failed to show persistent vaginal opening after 36 weeks, the mouse was sacrificed by avertin inhalation. Prior to sacrifice, a blood sample was obtained from the unconscious animal by cardiac puncture. The blood sample was centrifuged and serum collected and stored at -80°C for subsequent assay. After sacrifice, the uterus was removed and the wet

weight recorded. The uterus, renal capsule and graft were placed into fixative (3:1 100% ethanol: glacial acetic acid) for one hour and then transferred into 4% paraformaldehyde. After one hour, all sections were placed into 70% ethanol and stored at room temperature until they were histologically processed using haematoxylin and eosin staining (section 2.6.).

7.2.3. Hormone assays

7.2.3.1. Oestradiol

Concentrations of oestradiol in peripheral serum samples collected from the mice were determined using an affinity extraction procedure (Webb *et al.*, 1985). The minimum detectable value for this assay was 0.7pg/ml and the mean intra-assay CV was 8.2%. The intra-assay CV was 9.3%.

7.3. Results

Throughout the 37 weeks of study, all the animals showed no signs of ill health, except mouse 5 which was humanely killed 33 weeks after surgery. Mouse 5 had received ovarian cortex obtained from a lamb homozygous for the Booroola fecundity gene. After surgery, mouse 5 showed no signs of ill health and so the vaginal monitorings are included in the data. However, the data obtained at *post-mortem* were excluded.

7.3.1. Vaginal monitoring

For each mouse, the pattern of vaginal opening, which is a sign of oestrogenization, is shown in **Figure 7.1**. The timing of vaginal opening was not synchronized between animals and the length of time that the vagina remained open was not consistent between animals. For each

Figure 7.1. Twice weekly vaginal monitoring of ovariectomized severe combined immunodeficiency (SCID) mice which received ovine ovarian transplants from lambs that were either homozygous or non-carriers of the Booroola fecundity (Fec^B) gene. Mouse 1, 7, 8 and 9 received ovarian material from non-carriers and mouse 2, 3, 4 and 6 from homozygous carriers of the Fec^B gene. The sections coloured grey represent unrecorded data.

mouse, there was no inherent pattern observed in the time intervals between vaginal openings or in the length of time that the vagina remained open on each occasion (Figure 7.1.). The mean time interval from surgery to the first opening of the vagina was 11 ± 2 weeks and did not differ between homozygous and non-carrier ewes. Two of the animals (mouse 5 and mouse 7) failed to show any sign of the vagina being fully open at any time during the study, whilst mouse 1 and mouse 3 only exhibited full vaginal opening on one recorded occasion. Of the 9 animals, the remaining 5 mice exhibited full vaginal opening and did so on approximately 4 occasions during the study.

Prior to sacrifice, for all animals which exhibited an open vagina, a thin plastic pipette containing approximately 30-40 μ l of distilled water was placed into the vaginal opening and water gently infused before being withdrawn. This process was then repeated before the contents of the pipette were placed onto a clean slide and allowed to dry before the vaginal smear was viewed using the light microscope. The vaginal smears showed cornification of squamous epithelial cells indicative of that found in the normal oestrous cycle at oestrus (Table 7.1.). The vaginal smears obtained from mouse 2 showed only a few cornified cells which would be indicative of those observed during early oestrus.

7.3.2. Uterine weight

For all animals, the weight of the uterus was greater than would be expected in untreated ovariectomized mice (20mg; Mobbs, Chetney, Sinha and Finch, 1985; Gosden, personal communication). There was no significant difference in the mean (\pm s.e.m.) uterine weight between those animals in which the vaginas were closed, slightly open or open (79 ± 14 ,

Table 7.1. The effect of xenografting ovine ovarian cortex from Scottish Blackface lambs possessing the Booroola fecundity (Fec^B) gene upon the vagina and uterus of the ovariectomized SCID mouse.

Mouse	Genotype	Weeks in Host *	Oestradiol (pg/ml) ⁺	Vaginal Status	Vaginal Smear	Uterine Weight (mg)
1	Fec ⁺ Fec ⁺	37	42.01	closed		94
2	Fec ^B Fec ^B	15	105.61	open	early oestrus	61
3	Fec ^B Fec ^B	37	0.70	slightly open		55
4	Fec ^B Fec ^B	37	14.18	slightly open		184
6	Fec ^B Fec ^B	37	0.70	slightly open		120
7	Fec ⁺ Fec ⁺	37	0.70	closed		65
8	Fec ⁺ Fec ⁺	15	78.07	open	oestrus	221
9	Fec ⁺ Fec ⁺	22	38.05	open	oestrus	138

* Animals were sacrificed in week 37 after grafting if no persistent signs of vaginal opening were observed

Vaginal smears were classified as oestrus if there were cornified epithelial cells with no leucocytes

+ Minimum detectable value was 0.7pg/ml

120 \pm 37, 140 \pm 46 mg respectively), although the numbers in each category are small (n=2, n=3 and n=3 respectively).

7.2.3. Oestradiol

The concentrations of oestradiol in the serum samples are shown in **Table 7.1**. Animals which possessed an open vagina generally had higher peripheral oestradiol concentrations, although these failed to reach significance at the 5% level. Due to the low numbers of animals involved, there was no strong linear relationship ($r < 0.5$) between peripheral oestradiol concentration and uterine weight.

7.2.4. General morphology of the grafts

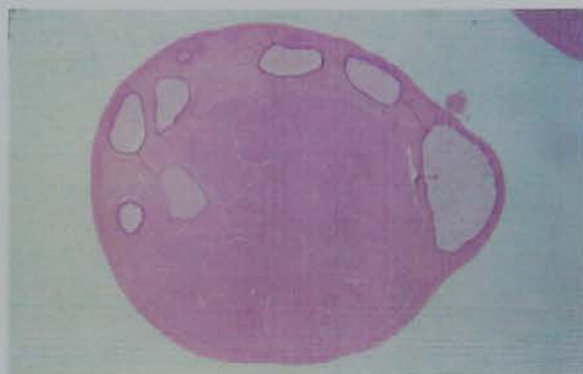
The grafts were still easily identified under the kidney capsule when the mice were sacrificed from weeks 15-37 (see **Figure 7.2.**). When the grafts were placed under the kidney capsule, they were as discrete pieces, but when the grafts were removed, the explanted tissue had increased in size and appeared as a single mass of white tissue. The 'junction' between the graft and the kidney tissue was well defined and the cells were not intermingled (see **Figure 7.2.**).

7.2.5. Follicular morphology

All stages of follicular development from primordial to antral were identified in the grafts (see **Figure 7.3.**). Thecal cells could be seen in both multilaminar and antral follicles. In many cases, the larger antral follicles were undergoing atresia with degeneration of the oocyte and the presence of pycnotic nuclei in granules as classified by Moor *et al.* (1978). Pycnotic bodies (dense mass) are indicative of cell death and occur as a result of shrinkage of nuclear material into a homogenous hyperchromatic mass. In all cases the grafts were anovulatory and no Graafian follicle or

Figure 7.2. Panel (a) is a normal sheep ovary showing the medulla, cortex, and follicles (x40 magnification). Panel (b) shows a haematoxylin and eosin-stained section of kidney with the ovine ovarian cortex outermost (x40 magnification). Panel (c) is ovarian cortex from a normal sheep, showing the development of preantral follicles. Similarly, panel (d) shows the presence of small preantral follicles in ovine ovarian cortex which was transplanted under the kidney capsule of the ovariectomized SCID mouse. Panel (e) shows a representative section of the 'junction' between the ovine ovarian graft and the kidney tissue of the mouse. Panel (c), (d) and (e) are photographed at x200 magnification.

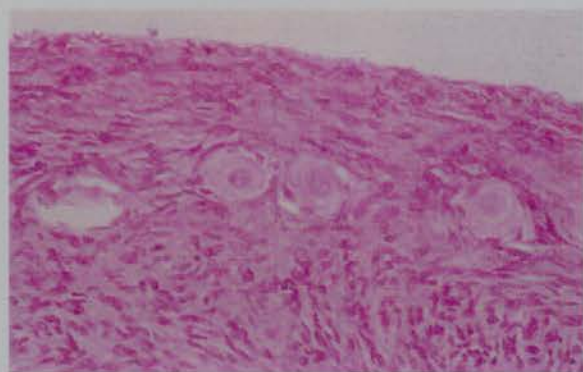
a



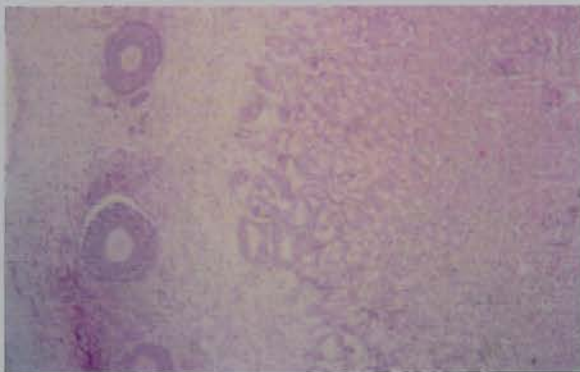
b



c



d



e

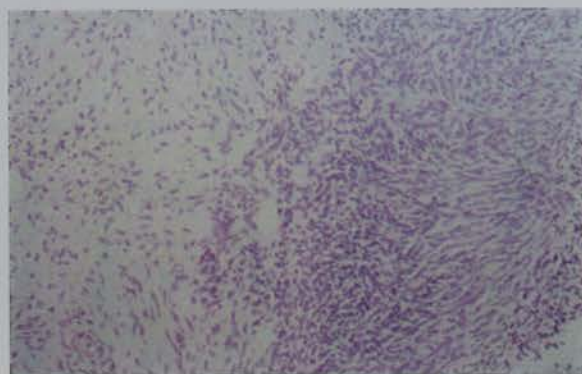


Figure 7.3. Panels (a-d) show haematoxylin and eosin-stained sections from ovine ovarian cortex transplanted under the kidney capsule of the ovariectomized SCID mouse. The presence of a range of follicular sizes within the grafts can be seen (see Table 7.2.). Panel (a) and (b) were photographed at x100, panel (c) and (d) at x 200 and x40 respectively.

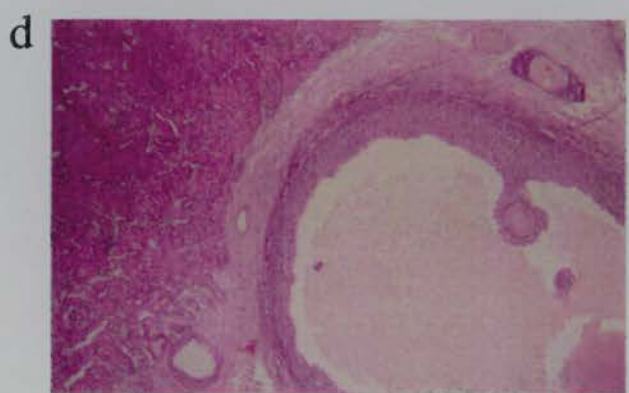
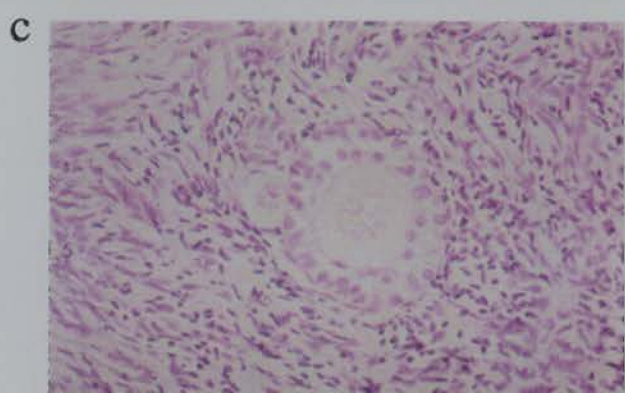
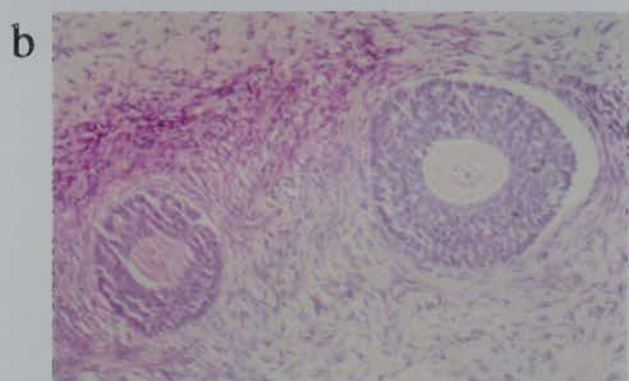
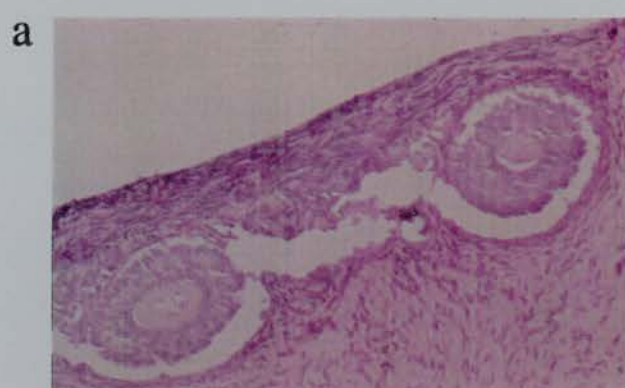


Table 7.2. The size distribution (mm) of ovine follicles which were obtained from lambs homozygous or non-carriers of the Booroola fecundity (Fec^B) gene and xenografted to ovariectomized SCID mice.

Mouse	Genotype	Follicle Diameter (mm)			
		<0.19	0.2-0.59	0.6-0.99	>1
1	Fec^+Fec^+	9	0	0	0
2	Fec^BFec^B	5	1	1	0
3	Fec^BFec^B	1	1	1	0
4	Fec^BFec^B	2	1	0	1
6	Fec^BFec^B	5	3	1	0
8	Fec^+Fec^+	3	3	0	2

Mice 7 and 9 were transplanted with ovarian material from Fec^+Fec^+ lambs. Follicles were observed on the surface of graft at autopsy, but the tissue did not remain intact after histological processing.

luteal structures were seen. Follicular diameters (mm) were measured using a calibrated graticule and categorized into various size ranges (Table 7.2.). In the xenografts, there were no follicles greater than 2mm in diameter (Table 7.2.).

7.3. Discussion

This study demonstrates that ovine ovarian cortex, containing primordial and small follicles, can be successfully transplanted under the kidney capsule of an immunologically deficient host mouse. The ovine follicles subsequently develop and progress to the antral stage.

It is rare for more than 50% of the total follicle population from mice to survive transplantation to other mice, unless vascular anastomosis is performed (Jones and Krohn, 1960; Green, Smith and Zuckerman, 1956). Primordial follicles are thought to be the only class of follicle which can survive the grafting process (Bland and Donovan, 1968), probably as a result of their peripheral location and lower requirement for metabolites (Gosden, 1992b). The adult ovine ovary contains between 12,000 and 86,000 primordial follicles (Cahill *et al.*, 1979; McNatty *et al.*, 1982) and so, on average, each square millimetre of ovarian cortex in the sheep should contain hundreds of follicles at the primordial and preantral stage, thus providing adequate numbers for transplantation.

Transplantation of small, or primordial, follicles isolated from stromal tissue would enable more control over the starting material such that the number and/or condition of follicles could be easily regulated. However, the presence of extraneous stromal tissue, as a prerequisite for normal follicular growth, is not fully understood. At present, however, it is not possible to easily dissect large numbers of small follicles from adult ovine ovaries (Chapter 5 and 6). Fetal ovarian material, a rich source of

primordial follicles, may be a more useful source of material in future experiments. It is now possible to obtain large numbers of primordial follicles, by manual dissection and centrifugation, from foetal bovine ovaries (Hulshof, Bevers, Van der Donk and Van den Hurk, 1991). Alternatively, small and primordial follicles from adult ovine ovarian cortex could be enzymatically dissociated as for the porcine ovary (Greenwald and Moor, 1989).

In the ewe, it takes approximately 6 months for a follicle to grow from the primordial stage to a Graafian follicle (Turnbull *et al.*, 1977; Cahill and Mauléon, 1980). It takes 130 days (approximately 19 weeks) for a follicle to reach antral size of approximately 0.2mm in diameter (Cahill and Mauléon, 1980), demonstrating the slow rate of follicular growth in sheep. In this study, the host vagina only opened after 8 weeks and during 19 weeks did so in six of the nine animals, although the length of time the vagina remained open was highly variable. This suggests that the rate of follicular growth may be similar even after transplantation into the SCID mouse. A persistently open vagina may be due either to the development of a single follicle which continuously produces oestradiol above the threshold necessary to stimulate vaginal opening, or due to a large turnover of follicles with each follicle becoming atretic, as is seen in aging anovulatory mice (Gosden, Laing, Flurkey and Finch, 1983). It is thought that growing follicles present in the ovarian cortex become atretic after transplantation, as a result of ischaemia, such that subsequent follicular development is due to the initiation of primordial follicle growth (Gosden, personal communication). It is at present unknown whether transplantation of ovine ovarian cortex to the kidney capsule of the ovariectomized SCID mouse elicits atresia of previously growing follicles, such that subsequent follicular development is due to the initiation of

primordial follicles. It would, however, appear unlikely that such follicles could remain in a 'suspended state' for 8 weeks before continuing oestradiol production (as assessed by the opening of the vagina). It has been estimated that up to 8 primordial follicles leave the primordial follicle 'pool' each day in the sheep (Turnbull *et al.*, 1977, Cahill and Mauléon, 1980; Driancourt *et al.*, 1985), such that 10-40 are visible on the surface of the ovary (Cahill *et al.*, 1979). Whilst the hierarchy of the follicle population within the xenograft did not represent that for the normal ovine ovary, the numbers of follicles initiated to grow may be dependent upon the quantity of transplanted material present (Browning and White, 1967).

This study showed that the xenografting technique of ovine ovarian cortex under the kidney capsule of the ovariectomized SCID mouse produced follicles which were able to form antra, but were unable to continue their development through to the final or Graafian stage. To date, only autografting experiments have produced follicles which are capable of ovulating (mice: Felicio, Nelson, Gosden and Finch, 1983; guinea-pigs: Bland and Donovan, 1968 and sheep: Goding, McCracken and Baird, 1967). The reason why xenografting fails to provide follicles capable of ovulating is poorly understood, but may involve an inappropriate hormonal signal from the host. Evidence for an imbalance in the hypothalamic-pituitary ovarian axis is indicated in this study by the irregular vaginal cycles in the ovariectomized SCID mice. Although further studies are required to confirm the hypothesis that anovulatory follicles occur due to a hypothalamic-pituitary imbalance, similar reports of persistent vaginal cornification, acyclic grafts and uterine hyperplasia have been reported in mice which received ocular isografts and were thought to be as a result of a failure in LH release (Browning and White, 1967). Alternatively, the failure to produce mature follicles capable of ovulating may be due to the

irregular growth of follicles rather than controlled waves of growth as in other species.

In the intact adult mouse, circulating concentrations of LH and FSH have been well characterized (Kovacic and Parlow, 1973; Murr, Geschwind and Bradford, 1973), showing that FSH ranges from 200-400ng/ml (with a peak concentration of 1,000ng/ml) and LH (Bronson and Stetson, 1973) ranges from a baseline of 2.5ng/ml to a peak of 40ng/ml (based on the NIAMDD standard in the rat gonadotrophin radioimmunoassay and verified for use in the mouse by Beamer, Murr and Geschwind, 1972). In lambs (Foster, Lemons Jaffe and Niswender, 1975a; Foster, Lemons Jaffe and Niswender, 1975b), FSH concentrations are similar to baseline concentrations obtained from adult ewes which are between 15-50ng/ml (Miller *et al.*, 1981). LH concentrations in lambs are higher than basal concentrations in the adult (Foster *et al.*, 1975a, Foster *et al.*, 1975b), but mean concentrations are similar due to the preovulatory gonadotrophin surge such that LH concentrations range between 0.4-120ng/ml (Goodman *et al.*, 1981). The transplanted ovine follicles, providing they have gained access to, and a dependence upon gonadotrophins, should initially receive adequate concentrations from the host, although LH pulse frequency and amplitude of the host may also be important in controlling subsequent ovine follicular development (see McNeilly, Crow, Brooks and Evans, 1992 for review). It is at present unknown how efficiently murine gonadotrophins can bind to the ovine receptor and the situation is further complicated by the fact that, despite the peripheral gonadotrophin concentrations in the mouse seeming to be adequate for the transplanted ovine material, the biological potencies of the gonadotrophins may differ between the species.

When the larger follicles fail to survive the grafting procedure as discussed earlier, the small and primordial ovine follicles which remain are known to secrete little or no oestrogen (see section 1.6.). In the intact ewe, during the breeding season, basal concentrations of oestradiol in the peripheral circulation are 2-4pg/ml and reach peak levels of 10pg/ml (Scaramuzzi and Land, 1978), most of which originate from the large or Graafian follicles (Hay and Moor, 1973; Moor, 1973). In the intact mouse however, basal peripheral oestradiol concentrations are at 1pg/ml and increase to peak values of 20pg/ml (Mobbs *et al.*, 1985). It is postulated that host gonadotrophin concentrations would therefore increase, due to the reduced level of negative feedback of oestradiol acting at the level of the host hypothalamus and/or pituitary gland. A reduction, or abolition, of the feedback effects of oestradiol, such as occurs in long-term ovariectomized mice can cause LH concentrations to increase to 200ng/ml (Mobbs *et al.*, 1985) and FSH to 700ng/ml (Kovacic and Parlow, 1973). The circulating levels of gonadotrophins in ovariectomized SCID mice which have received ovine ovarian transplants are at present unknown and should be an interesting avenue for further work.

Recent work using the ovariectomized SCID mouse model and transplantation of feline ovarian cortex under the kidney capsule, caused vaginal cornification and endometrial hyperplasia of the host within a month, approximately the normal time period for follicles to reach maturity to the Graafian stage for this species (Gosden, 1992b). In this study, the explanted ovine follicles in some animals caused vaginal opening and cornification of the vagina, both observations being indicative of oestrogenization (Murray and Stone, 1989), but the normal time course taken to reach the Graafian stage could not be determined since Graafian follicles did not develop. The uterine weight from all mice which had

been transplanted with ovine ovarian cortex were elevated compared to castrate (Holinka, Hetland and Finch, 1977; Gosden, personal communication), or intact (Kovacic and Parlow, 1973), animals and support the results of the vaginal monitoring in that oestrogenization occurred in some cases. For animals which exhibited vaginal closure, the weight of the uterus was still higher than in castrate animals. The reason for this is unlikely to be due to a difference between the uterus and the vagina in their sensitivity to oestradiol and is more probably as a result of the stimulatory effect of circulating androgen on the uterus. Chronic exposure to physiological doses of androgens have been reported to depress uterine weights in rats (Rocheffort and Garcia, 1968). This low dose androgen-induced inhibition of increases, or actual decreases, in uterine weight may operate either directly through the androgen receptor, or through interaction at the level of the hypothalamic-pituitary glands. In contrast, high doses of androgens are reported to stimulate uterine growth in the rat (Lerner, Hilf, Turkheimer, Michel and Engle, 1966), probably by androgen binding to the oestrogen receptor to elicit an oestrogen-like response (Rocheffort, Lignon and Capony, 1972; Ruh, Wassilak and Ruh, 1975). There was, however, considerable variation between animals which received xenografts in the subsequent oestradiol concentrations and uterine weights obtained, although it seems unlikely that this variation between animals is due to the age differences in the mice at sacrifice (Mobbs *et al.*, 1985) and is more probably due to the variation in follicular growth within the transplanted material. The increase in uterine weight above that for a castrate animal may be due to both an accumulation of uterine fluid and/or an increase in cell number.

Deansley (1956) and Parkes (1956) have described and illustrated the range of normal structures in autografts which may be found in ovarian

Table 7.3. The advantages and disadvantages of the ovariectomized SCID mouse model as a tool to study development of the sheep follicle.

Advantages	Disadvantages
<ul style="list-style-type: none"> • Very small amount of starting material required. 	<ul style="list-style-type: none"> • Unknown number of primordial follicles in the starting material. Dissection of primordial follicles not yet possible on a large scale.
<ul style="list-style-type: none"> • Scientific protocol simple to perform. 	<ul style="list-style-type: none"> • Specialist husbandry and equipment are required for the management of SCID mice.
<ul style="list-style-type: none"> • Follicles survive transplantation and look histologically normal. 	<ul style="list-style-type: none"> • Maintenance of the SCID mice labour intensive.
<ul style="list-style-type: none"> • Allows long term <i>in vivo</i> studies of follicular development to be performed. 	<ul style="list-style-type: none"> • Taking serial blood samples from the same mouse is not possible.
<ul style="list-style-type: none"> • Vaginal openings of the SCID mice are a quick, reliable bioassay of follicular oestrogenization. 	<ul style="list-style-type: none"> • Development to the Graafian stage does not seem possible.

tissue a year or more after grafting. The long time period required for ovine folliculogenesis to occur may however provide additional complications to the success of using the ovariectomized SCID mouse xenografts as an *in vivo* model. Vaginal opening and cornification of the squamous epithelial cells is a sensitive indicator of oestradiol activity in acute studies, but chronic exposure to oestradiol impairs the ability of the vaginal epithelium to cornify in response to continuous exposure (Adler and Nelson, 1988; Jesionowska, Karelus and Nelson, 1990). Incidences of vaginal acyclicity in the presence of high oestrogen concentrations and normal ovarian function have been reported in mice when oestrogens were artificially raised to 120-160pg/ml (Adler and Nelson, 1988). Further complications arising from long-term grafting experiments have arisen and questioned the ability of the follicles to remain viable and resist premature recruitment (Gosden, 1990b). Other points to consider in the use of the SCID mouse xenograft model specifically applied to the xenografting of ovine ovarian material are listed in **Table 7.3**.

In conclusion, the inability of the ovariectomized SCID mouse model to maintain ovine follicular development through to the final stages of differentiation is a limitation in studying the follicular growth patterns. However, the ovariectomized SCID mouse model may prove beneficial as a tool to investigate factors involved in the initiation of follicular growth. The ovariectomized SCID mouse xenograft model, however, may prove to be a useful *in vivo* tool to investigate the mechanisms controlling folliculogenesis between genotypes of differing fecundity such as the Booroola Scottish Blackface sheep. However, in this current study genotypic differences in follicular development could not be ascertained and larger numbers of animals will be required in future experiments.

CHAPTER 8

Investigation of genotypic differences in peripheral IGF-1 concentrations

8.1. Introduction

The primary role of gonadotrophins in the regulation of ovarian follicle growth and differentiation has been well established (section 1.5.2.). IGF-1 is thought to be an amplifier of, and stimulated by, gonadotrophins, since it can modulate growth, differentiation and steroidogenesis of follicles (see section 1.6.3.). In sheep, IGF-1 gene expression and translation in relation to the stage of oestrous cycle is not known, although in rats it increases between proestrus and oestrus, suggesting IGF-1 is an important mediator, or modulator, for the growth promoting and differentiating effects of gonadotrophins (Carlsson, Carlsson and Billig, 1989). If IGF-1 is an important regulator in the control of ovine folliculogenesis, it is plausible that the Booroola fecundity gene may be operating through differences in peripheral FSH concentrations and/or differential secretion of IGF-1. Support for this hypothesis is seen in cattle selected for twinning, where IGF-1 concentrations in serum and follicular fluid are higher (Echternkamp, Spicer, Gregory, Canning and Hammond, 1990). Furthermore, IGF-1 concentrations appear to be genetically determined in several other species (man: Merimee, Zapf and Froesch, 1982; mouse: Blair, McCutcheon, Mackenzie, Ormsby, Siddiqui, Breier and Gluckman, 1988 and swine: Buonomo, Lauterio, Baile and Campion, 1987).

The aim of this study was to investigate whether differences in ovulation rate between genotypes of the Booroola fecundity gene may be related to differences in mean peripheral IGF-1 concentrations.

8.2. Materials and Methods

8.2.1. Animals

The animals used in Experiment 1 were the same as described in section 3.2.1. The F₂ Booroola Scottish Blackface ewes were classified as either the Fec^BFec^B (n=9), Fec^BFec⁺ (n=9) or Fec⁺Fec⁺ (n=9) genotype and were 2-4 years of age.

The animals used in Experiment 2 were the same as described in section 4.2.1. The F₂ Booroola Scottish Blackface ewes were classified as either the Fec^BFec^B (n=10), Fec^BFec⁺ (n=8) and Fec⁺Fec⁺ (n=8) genotype and were 2-4 years of age.

8.2.2. Experimental protocol

8.2.2.1. Peripheral IGF-1 during the oestrous cycle in the F₂ population (Experiment 1)

The experimental protocol has been described previously (section 3.2.2., Experiment 4). Briefly, blood samples were obtained by jugular venepuncture from day -3 until day 13, with day 0 being the day of behavioural oestrus. Laparoscopy was performed during the luteal phase (days 9-12) to determine the ovulation rate.

8.2.2.2. Effect of ovariectomy on peripheral IGF-1 in the F₂ population (Experiment 2)

The experimental protocol has been described previously (section 4.2.2., Experiment 2). Briefly, during the breeding season F₂ Booroola Scottish Blackface ewes were synchronised using vaginal progestagen sponges for 12 days. All ewes were run with a raddled ram, checked twice daily for signs of oestrus and sampled twice daily by jugular venepuncture from the first day of sponge removal (day -2) until day 7 of the oestrous cycle. On day 7, all ewes were ovariectomized as described in section 2.2.3. The day of ovariectomy was now termed day 0. Blood sampling by jugular venepuncture was continued for 14 days post-ovariectomy.

8.2.3. *Hormone assays*

8.2.3.1. IGF-1

Concentrations of IGF-1 in peripheral serum samples were determined in duplicate as validated and described in sections 2.3.5. and 2.3.5.1. respectively. The minimum detectable value was 17.8ng/ml and the intra-assay and inter-assay CV were 9.9% and 9.0% respectively.

8.2.3.2. Statistical analyses

IGF-1 concentrations in the daily serum samples from each animal are expressed as arithmetic means. These daily within animal means were then used to obtain a group arithmetic mean (\pm s.e.m.). All normally distributed data, as assessed from the Statworks package on the Apple Macintosh, are expressed as arithmetic means (\pm s.e.m.) and compared by Student's t test using the Minitab programme on the Apple Macintosh. Individual ovulation rates are expressed as a group arithmetic mean (\pm

s.e.m.) and compared by Student's t test. The influence of bodyweight upon IGF-1 levels and ovulation rates was not significant and was therefore excluded from further analyses.

8.3. Results

8.3.1. Experiment 1

8.3.1.1. IGF-1

Mean (\pm s.e.m.) concentrations of IGF-1 (from day -3 to day 13 of the oestrous cycle) for each genotype are shown in **Figure 8.1**. There were no significant differences between homozygous, heterozygous and non-carrier ewes in mean concentration of IGF-1 from day -3 to day 13 (**Table 8.1**). For all genotypes, there was a significant ($P < 0.01$) effect of time upon the concentrations of IGF-1, with concentrations increasing during the follicular phase of the oestrous cycle.

8.3.1.2. Ovulation rates

During the breeding season the mean ovulation rates differed significantly ($P < 0.001$) between homozygous, heterozygous and non-carrier ewes (4.4 ± 0.4 , 2.4 ± 0.2 and 1.6 ± 0.2 respectively).

8.3.2. Experiment 2

8.3.2.1. IGF-1

Mean (\pm s.e.m.) peripheral concentrations of IGF-1 for each genotype are shown in **Figure 8.2**. There was no significant difference between genotypes in mean IGF-1 concentrations before ovariectomy, between days -2 to 6 of the oestrous cycle (**Table 8.1**). After ovariectomy mean peripheral IGF-1 concentrations between days 0 to 14 did not differ

Figure 8.1. Mean (\pm s.e.m.) serum IGF-1 concentrations (ng/ml), from the three genotypes of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=9 per group), from day -3 to day 13 of the oestrous cycle, with day 0 being the day of oestrus (Experiment 1). Daily serum samples for individual ewes, from each genotype, are expressed as an arithmetic mean. These daily within animal means are used to calculate a group arithmetic mean.

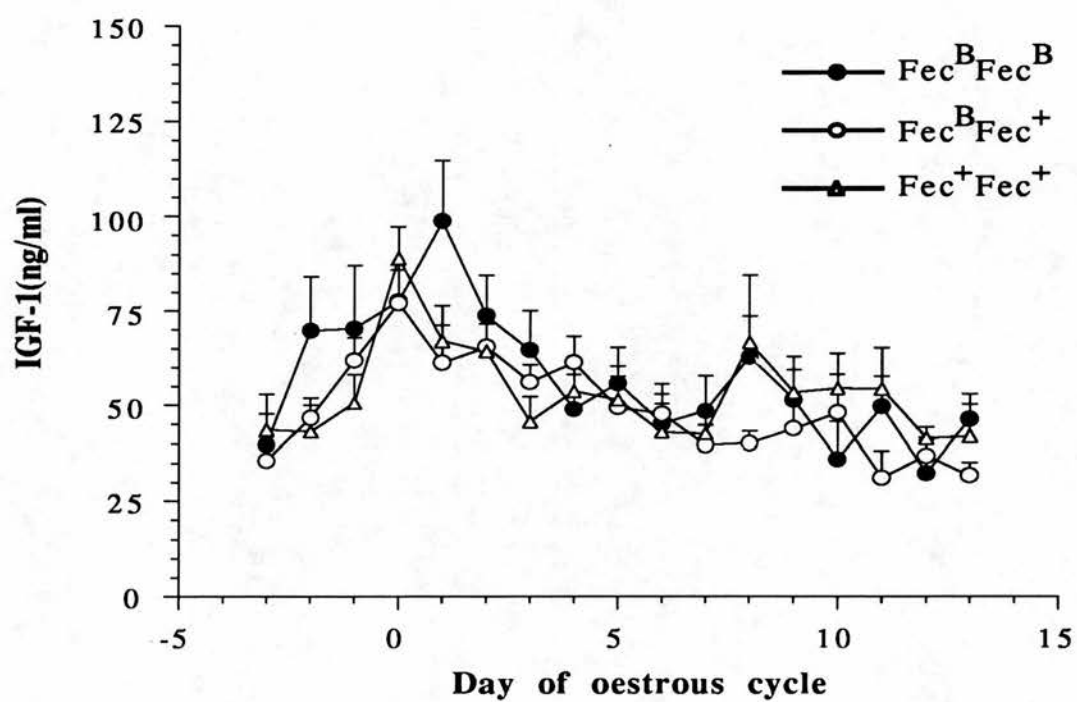


Figure 8.2. Mean (\pm s.e.m.) serum IGF-1 concentrations (ng/ml), from the three genotypes, homozygous, heterozygous and non-carriers of the F₂ population possessing the Booroola fecundity (Fec^B) gene (n=10, n=8 and n=8 respectively), from day -9 to day 14. Day 0 was the day of ovariectomy, equivalent to day 7 of the oestrous cycle (Experiment 2). Daily serum samples for individual ewes, from each genotype, are expressed as an arithmetic mean. These daily within animal means are used to calculate a group arithmetic mean.

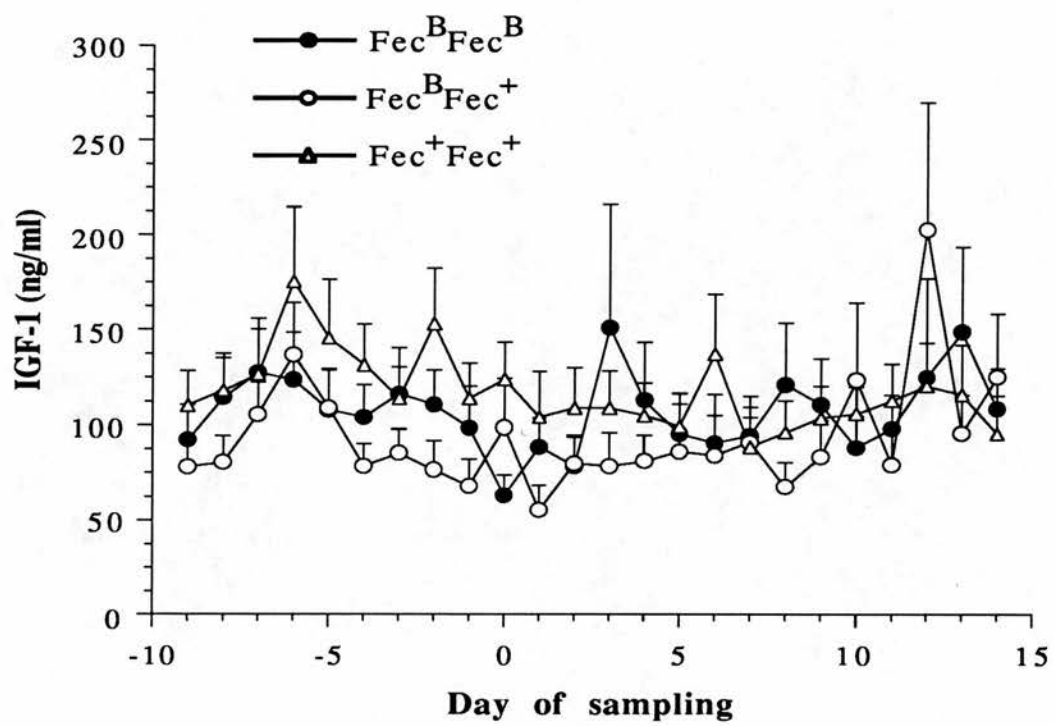


Table 8.1. Mean (\pm s.e.m.) IGF-1 concentrations (ng/ml) in daily blood samples collected from day -3 until day 13 of the oestrous cycle (Experiment 1) and pre- and post- ovariectomy (Experiment 2) in the F₂ population of Scottish Blackface ewes with and without the Fec^B gene.

Group	Genotype		
	Fec ^B Fec ^B	Fec ^B Fec ⁺	Fec ⁺ Fec ⁺
Oestrous cycle	56.7 \pm 5.6 (n=9)	49.51 \pm 4.2 (n=9)	53.5 \pm 3.2 (n=9)
Pre-ovariectomy	110.7 \pm 17.3 (n=10)	91.0 \pm 13.6 (n=8)	132.2 \pm 16.5 (n=8)
Post-ovariectomy	105.1 \pm 22.0 (n=10)	95.5 \pm 20.0 (n=8)	108.6 \pm 15.8 (n=8)

between genotypes (Table 8.1.). For each genotype, mean peripheral IGF-1 concentrations before ovariectomy were not significantly different from the mean concentrations after ovariectomy.

8.3.2.2. Ovulation rates

Mean ovulation rates at ovariectomy were significantly different ($P < 0.05$) between homozygous, heterozygous and non-carrier ewes (3.6 ± 0.7 , 2.3 ± 0.2 and 1.3 ± 0.2 respectively).

8.4. Discussion

These data suggest that peripheral concentrations of IGF-1 are not related to genotypic differences in ovulation rate. In the F₂ population of ewes (Experiments 1 and 2), significant differences in genotypic mean ovulation rates were observed, whereas mean peripheral IGF-1 concentrations did not differ between genotypes.

The role of IGF-1 in controlling ovine follicular growth and differentiation is not fully understood. However, ovine granulosa cells are known to possess IGF-1 type receptors (Monget, Monniaux and Durand, 1989) and proliferate in response to physiological concentrations of IGF-1 *in vitro* (Webb and McBride, 1991). The role of IGF-1 in promoting proliferation and differentiation and/or its interaction with FSH are known to be highly dependent upon both the stage of development, and state of atresia, of the ovine follicle (Monget *et al.*, 1989; Monniaux and Pisselet, 1992). In contrast, IGF-1 concentrations in the follicular fluid are found to increase as follicular diameter increases (cattle: Echtenkamp *et al.*, 1990; pigs: Hammond, Hsu, Klindt, Tsang and Downey, 1988; mares: Spicer, Tucker, Henderson and Duby, 1991). It has also been found in mares

that peripheral IGF-1 concentrations are greater than, and significantly correlated with, follicular fluid IGF-1 concentrations (Spicer *et al.*, 1991).

Whilst it is known that the number of IGF-1 receptors on ovine antral follicles is not affected by the size of the follicle (Monget *et al.*, 1989), it has yet to be determined whether follicular fluid concentrations of IGF-1 are positively related to follicle diameter in sheep. This may be of importance, since follicles from Booroola fecundity gene carriers ovulate at a smaller diameter than non-carriers (Scaramuzzi *et al.*, 1983). Alternatively, rather than absolute differences in follicular fluid concentrations of IGF-1 being important, there may be genotypic differences in the capacity of follicles to respond to and/or produce IGF-1. Recent evidence has found that effectiveness of IGF-1 in promoting growth and cyto-differentiation, as assessed by induction of progesterone secretion, varies between granulosa cells from small and large follicles (Monniaux and Pisselet, 1992). These authors suggest there may be at least two populations of granulosa cells within ovine follicles that respond differently to IGF-1. It could be hypothesized that genotypic differences in ovulation rate which are observed in Booroola Scottish Blackface ewes may involve earlier follicular maturation which could be as a result of genotypic differences in the proportions of these subpopulations of granulosa cells and local effects of IGF-1. Monniaux and Pisselet (1992) showed that IGF-1 acts synergistically with FSH to alter ovine granulosa cell cytodifferentiation, as indicated by an increase in progesterone production. This is an interesting area for further investigation, since follicles from prolific strains are thought to 'mature' at reduced diameters compared with follicles from animals of lower prolificacy (see section 1.4.3.).

In addition, our previous work (Chapter 3) showed that FSH concentrations were higher during both the oestrous cycle and seasonal anoestrus in homozygous carriers of the Booroola fecundity gene compared to non-carriers. If, within each genotype, FSH regulates and potentiates IGF-1 production, one may expect peripheral IGF-1 concentrations to mimic those genotypic differences already observed for FSH concentrations. In this study, peripheral IGF-1 concentrations did not differ between genotypes and so raises further questions as to whether local mechanisms could be involved. It may be that, within each genotype, FSH potentiates IGF-1 production within the ovary to the same degree, such that homozygous carriers, with higher peripheral FSH concentrations, do have higher ovarian IGF-1 concentrations than non-carriers. This effect would probably be masked by the larger IGF-1 output into the peripheral circulation by the liver. Alternatively, in response to genotypic differences in FSH, there may be an alteration in local IGF-1 production within the ovary, such that peripheral IGF-1 concentrations appear not to be affected by genotype, similar to genotypic differences in oestradiol production, where homozygous carriers produced less on a per follicle basis compared to non-carriers, and yet peripheral levels do not differ (McNatty *et al.*, 1985a; McNatty *et al.*, 1986a). It is interesting to note therefore, that oestradiol is a potent stimulator of IGF-1 action (Hammond *et al.*, 1985), thereby providing another possible area of control. IGF-1 concentrations within follicular fluid were not measured in this study, but would be a useful avenue for further study. Falconer, Bindon, Piper and Hillard (1989), using an acetic acid extraction procedure, compared follicular fluid concentrations of IGF-1 in ewes possessing the Booroola gene with Merinos of a different strain and reported IGF-1 concentrations to be similar. These authors also found IGFBP was significantly higher in

control Merinos compared to Booroola carriers of the fecundity gene. The findings of these results have yet to be confirmed and the hypothesis that the enhanced prolificacy of the Booroola is not due to increased IGF-1 production, but due to potentiation of IGF-1 action by enhanced binding protein awaits further investigation.

As mentioned previously, the liver is a major synthesizer of IGF-1. However, IGF-1 does not just act locally within the liver, but enters the circulation to have an endocrine action (D'Ercole, Stiles and Underwood, 1984; Hodgkinson, Spencer, Bass, Davis and Gluckman, 1991). In addition ovarian hormones may influence the biosynthetic capability of the liver. Oestradiol administered to ovariectomized cattle have resulted in an increase in IGF-1 levels, implicating oestrogenic involvement in hepatic IGF-1 production (Richards, Wettemann, Spicer and Morgan, 1991) despite pharmacological doses being used. Furthermore, in cattle, ovariectomy on day 7 of the cycle results in an acute decrease in IGF-1 concentrations (Richards *et al.*, 1991), although no long-term effect on basal concentrations have been reported. Interestingly, in this study, ovariectomy did not alter peripheral mean IGF-1 concentrations and suggests that the ovary is not a major source of IGF-1. However, increases in peripheral IGF-1 concentrations did occur during the follicular phase (Experiment 1). It is unknown whether these changes are functionally important, but effects of steroids on IGF-1 and IGFBP production and clearance warrants further study.

It is unknown whether the lack of an effect of ovariectomy on IGF-1 concentrations in sheep (Experiment 2), in contrast to the results of Richards *et al.* (1991) in cattle, is a true species difference, or is as a result of the different methodologies to remove IGF-binding proteins (IGFBPs). The work of Richards *et al.* (1991) involves extraction using an acid ethanol

(AE) protocol, whereas we used an extraction procedure based upon acetone, ethanol, acetic acid (AEA) see section 2.3.5.1. for validation. It is at present unknown why mean IGF-1 concentrations, from day -3 until day 13 of the oestrous cycle, are higher than those observed pre-ovariectomy, that is, from day -2 until day 7. This apparent discrepancy may be a true biological effect, although it may involve the length of time the sample, (extracted or unextracted) has been stored at -20°C prior to radioimmunoassay, since attempts to reassay extracted samples result in an apparent reduction in the IGF-1 concentrations. Clearly further investigation is required to determine suitable storage conditions for 'EAA-extracted' IGF-1 samples. However, acid ethanol (AE) extraction procedures in particular, have been criticised since this methodology of extraction can lead to an over-estimation in the immunopotency in serum due to the interference of IGFBPs (Breier *et al.*, 1991). Identification of the biological sources and mechanism of action of the IGFs has also been complicated, or misinterpreted, by discrepancies in quantitative assay procedures between different laboratories such that, for example, AE extraction methods can differ by 54% (Echternkamp *et al.*, 1990). It is at present unknown if AE extraction results in a consistent over-estimation of IGF-1 concentrations, such that the absolute levels differ, but relative differences between samples are still maintained.

Despite these worrying discrepancies between the various methodologies, across species a number of trends seem to be apparent. In this study, IGF-1 concentrations were found to increase during the follicular phase of the oestrous cycle in agreement with other authors (sheep: Spicer and Zavy, 1992; rats: Carlsson *et al.*, 1989). Further work will be required to determine if hepatic IGF-1 is involved in the regulation of

follicular development, or whether IGF-1 of ovarian origin is more important.

Spicer and Zavy (1992), used an EA extraction procedure and compared peripheral IGF-1 concentrations in ewes of the same age, but in breeds with different ovulation rates. They found no differences in IGF-1 concentrations between Dorset x Rambouillet, Finnish Landrace x Rambouillet and Booroola Merino x Rambouillet ($Fec^B Fec^B$) ewes (ovulation rate: 1.4, 1.9 and 2.5 respectively). The results of the current experiment also found no differences in peripheral IGF-1 concentrations between Booroola Scottish Blackface genotypes despite three fold differences in ovulation rate between homozygous carriers and non-carriers, and in three genotypes which are more closely related genetically. Both sets of data suggest that peripheral IGF-1 is not primarily responsible for the genotypic differences in ovulation rates observed both within or between breeds.

To add further complexity, IGF-BPs (see Sara and Hall, 1990 for review), have been shown to be important in modulating follicle differentiation either directly (Ui, Shimonaka, Shimasaki and Ling, 1989; Ricciardli, Hernandez, Hurwitz, Kokia, Rosenfeld, Schwander and Adashi, 1991; Bicsak *et al.*, 1991; Howard and Ford, 1992), or indirectly through regulation of growth factor availability (Bicsak *et al.*, 1991). Such IGF-BPs may be in turn regulated by the growth factor itself (Grimes and Hammond, 1992). It is unknown whether the absolute concentration of IGFBPs vary between genotypes possessing the Booroola fecundity gene, or whether the proportions of IGFBPs are differentially altered at crucial time points during the oestrous cycle. Recently, the activity of IGFBPs was found not to differ during the oestrous cycle, or between Finnish Landrace ewes which differed in ovulation rates (Spicer, Hanrahan, Zavy and Enright, 1993).

In conclusion, peripheral concentrations of IGF-1 in serum do change significantly during the oestrous cycle in the ewe, suggesting acute regulation of IGF-1 production by the ovary, which is itself not the main source of IGF-1 production. However, no genotypic differences in peripheral IGF-1 production could be found thus questioning the role of peripheral IGF-1 in regulating ovulation rate.

CHAPTER 9

Follicular fluid concentrations and ovarian secretion rates of inhibin and oestradiol

9.1. Introduction

The secretion of FSH by the pituitary gland is under the dual inhibitory control of oestradiol and inhibin (Martin *et al.*, 1988; Webb, 1988). The follicular and luteal phases of the oestrous cycle are characterized by peripheral plasma concentrations of FSH which are inversely correlated with oestradiol and inhibin (McNeilly and Baird, 1989; Findlay, Clarke and Robertson, 1990; McNeilly *et al.*, 1989), consistent with the hypothesis that both hormones contribute to the control of FSH release (see Baird *et al.*, 1991 for review).

Ovine inhibin is a glycoprotein hormone secreted by ovarian follicles (section 1.5.2.) which may have direct actions in the control of follicular growth and development (Woodruff, Lyon, Hansen, Rice and Mather, 1990; see Burger, 1989; Mather *et al.*, 1992; Baird *et al.*, 1991 for reviews). The precise mechanism involved in the control of the number of follicles that ovulate has yet to be resolved (see section 1.4.1.) but may involve the ability of inhibin and/or oestradiol to inhibit the secretion of FSH, such that follicles which are less sensitive to the decline in FSH concentrations after luteolysis continue to develop, whilst those follicles which are more sensitive, become atretic (Baird *et al.*, 1991).

Since the secretion of inhibin reflects the total population of antral follicles (section 1.2.3.) and oestradiol reflects the number of dominant follicles (Campbell *et al.*, 1991; see Baird *et al.*, 1991 for review), it is postulated that differences in these two hormones may occur in breeds

which differ in prolificacy. Our previous studies have shown that FSH is significantly higher in homozygous ewes compared to heterozygous or non-carrier ewes, in both intact and ovariectomized animals (Chapters 3 and 4), but it is at present unknown whether peripheral inhibin concentrations and/or secretion rates differ between these genotypes.

The aim of this study was to investigate the relationship between inhibin and oestradiol in our populations of Booroola Scottish Blackface ewes, to determine if there was an association between the presence of the fecundity gene and both inhibin and oestradiol secretion.

9.2. Materials and Methods

9.2.1. Animals

The animals used in Experiment 1 were obtained from backcross Booroola Scottish Blackface population and classified as being either $Fec^B Fec^+$ (n=6) or $Fec^+ Fec^+$ (n=6) genotype as described previously in Chapter 6.

The animals used in Experiment 2 were obtained from the F_2 Booroola Scottish Blackface population and classified as being either $Fec^B Fec^B$ (n=10), $Fec^B Fec^+$ (n=8) or $Fec^+ Fec^+$ (n=8) genotype as described previously in Chapter 4.

9.2.2. Experimental protocol

9.2.2.1. Follicular fluid concentration of oestradiol and inhibin in the backcross population (Experiment 1)

The follicles used in this experiment were obtained from ewes (section 6.2.1.) which were ovariectomized (section 2.2.3.) during seasonal anoestrus (July). All follicles >1mm in diameter were dissected and

immersed into liquid nitrogen before being stored at -80°C until follicular fluid collection as described in section 2.4.

9.2.2.2. Ovarian secretion and follicular fluid concentration of oestradiol and inhibin in the F₂ population (Experiment 2)

The follicles used in this study were obtained from ewes during the breeding season in December (see section 4.2.1.). Briefly, the oestrous cycles of F₂ Booroola Scottish Blackface ewes were synchronised using vaginal progestagen sponges (Veramix, Crowley) for 12 days. All ewes were run with a raddled ram and checked twice daily for signs of oestrus (day 0) following sponge withdrawal. On day 7, a single blood sample was obtained at 09.00 hours by jugular venepuncture from all ewes which were then surgically prepared and anaesthetized as described in section 2.2.2. Ovarian vein blood from both ovaries was collected into separate sterile graduated tubes for 10 minutes. Ovarian vein blood flow per minute was calculated and corrected for haematocrit (Collett, Land and Baird, 1973). Mean (\pm s.e.m.) haematocrit was $26\% \pm 0.6$ ($n=12$) and is similar to that reported by Spector (1956). All ewes were then ovariectomized as described in section 2.2.3. and all follicles which were $>1\text{mm}$ in diameter were dissected, immersed into liquid nitrogen and then stored at -80°C until follicular fluid collection (section 2.4.1.). Follicular fluid hormone concentrations were calculated as described in section 2.4.2.

9.2.3. Hormone assays

9.2.3.1. FSH

Concentrations of FSH in peripheral serum samples were determined in duplicate by radioimmunoassay (see section 2.3.2.). The minimum

detectable value was 0.99ng/ml. The mean intra-assay and inter-assay CV were 7.2% and 10.0% respectively.

9.2.3.2. Oestradiol

Concentrations of oestradiol in peripheral serum were determined by radioimmunoassay incorporating an affinity chromatography extraction procedure (see section 2.3.3.). The minimum detectable value was 0.7pg/ml and the mean intra-assay and inter-assay CV were 8.2% and 3.0% respectively.

Concentrations of oestradiol in follicular fluid were determined by radioimmunoassay (see section 2.3.3.). The minimum detectable value was 7.8pg/ml and the intra-assay and inter-assay CV were 8.5% and 9.3% respectively.

9.2.3.3. Inhibin

Concentrations of dimeric inhibin in follicular fluid were determined using a modified version of the two site immunoradiometric assay (IRMA) described by Knight, Groome and Beard, 1991; Knight, Muttukrishna, Groome and Webley (1992). Modifications included the addition of sodium dodecyl sulphate (SDS; 0.1% final concentration) to each assay tube containing diluted standards or test samples, 30 minutes before the addition of labelled and unlabelled antibodies. In addition, 20% horse serum was added to the diluent containing the labelled and unlabelled antibodies. Finally, the concentration of DARS precipitating serum was raised to 20% and the NRS was omitted from this reagent. IRMA has minimal cross-reactivity with both activin and free biologically inactive, α -subunit. The inhibin standard used was a highly purified preparation of molecular weight (Mr) 32,000 bovine inhibin (Knight, Castillo, Glencross,

Beard and Wrathall, 1990). The minimum detectable value was 500 pg/ml. The mean intra-assay and inter-assay CV were 4.1% and 8.4% respectively.

It was not possible to determine concentrations of inhibin in serum from either ovarian venous or jugular venous blood by IRMA. High concentrations of ovine immunoglobulin (Ig) G present in undiluted serum samples saturated the binding capacity of the double anti-sheep IgG precipitating serum used in IRMA, making it impossible to precipitate the ovine capture antibody (Knight, personal communication). Inhibin concentrations in serum were therefore determined by the radioimmunoassay of McNeilly, Swanston, Crow, Tsonis and Baird (1989), which detects all forms of inhibin and free α -subunit on an equimolar basis (McNeilly *et al.*, 1989). The inhibin standard used was a highly purified preparation of porcine 1-26 amino acid sequence of the N-terminus of the α -chain of 32kDa inhibin peptide. The minimum detectable value was 63pg/ml and the intra-assay and inter-assay CV were 8.8% and 9.5% respectively.

9.2.3.4. Statistical analyses

For each animal the secretion rate of inhibin was calculated as shown below,

$$\text{Secretion} = \frac{\text{ovarian vein concentration} - \text{jugular vein concentration}}{\text{Rate of blood flow}} \times$$

The secretion rate of inhibin for each animal are expressed as a group arithmetic mean (\pm s.e.m.) for each genotype. All normally distributed data, as assessed from the Statworks package on the Apple Macintosh, are expressed as arithmetic mean (\pm s.e.m.) and compared by Student's t test using the Minitab programme on the Apple Macintosh. Follicular fluid

concentrations of oestradiol and inhibin exhibited a skewed distribution and values were logarithmically transformed prior to statistical analyses. These logarithm means were expressed as individual geometric means (\pm s.e.m.) which were then used to calculate a group geometric mean (\pm s.e.m.). Correlation coefficients (r) were performed on transformed data, and frequency distributions by Chi-square (X^2) analyses were calculated using the Minitab programme on the Apple Macintosh.

9.3. Results

9.3.1. Experiment 1

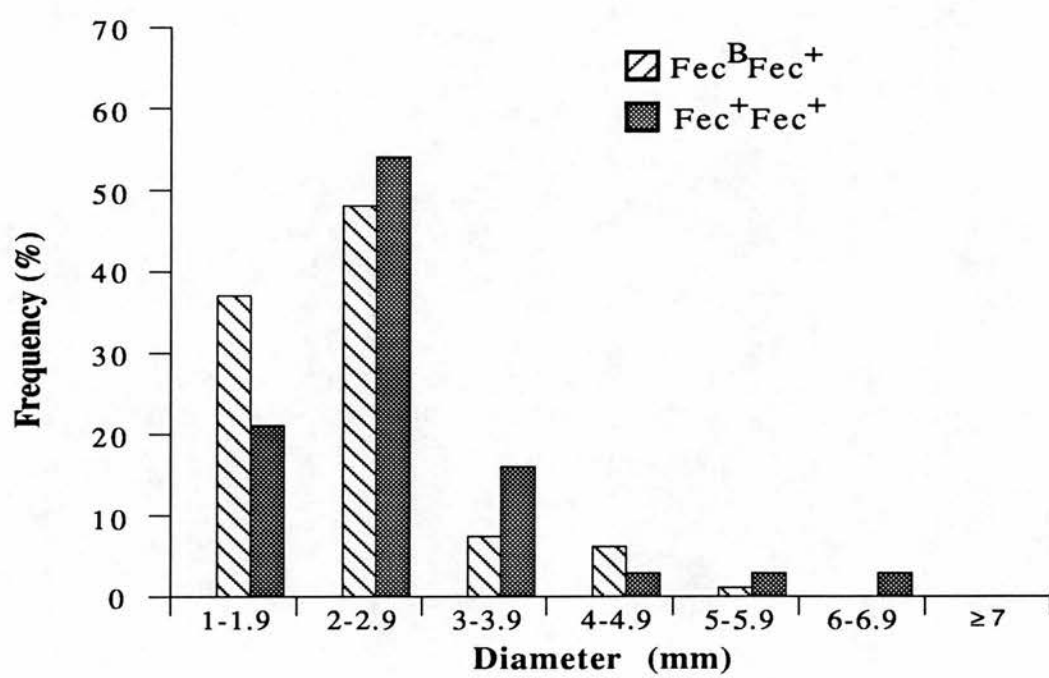
9.3.1.1. Follicle distribution

The total number of follicles per ewe ($>1\text{mm}$ in diameter) did not differ between heterozygous and non-carrier ewes (17 ± 2 and 15 ± 2 respectively). The distribution (% frequency) of follicles based on diameter is shown in the **Figure 9.1**. The proportion of follicles within each size range was not significantly different between genotypes.

9.3.1.2. Follicular fluid oestradiol

Geometric mean oestradiol concentration in follicular fluid, irrespective of oestrogenic status of the follicle, did not differ between heterozygous and non-carrier ewes (7.89 ± 1.05 , 5.89 ± 3.63 ng/ml respectively). Follicles having follicular fluid oestradiol concentrations $\geq 50\text{ng/ml}$ were designated oestrogen-active, and the remaining follicles designated oestrogen-inactive (McNatty *et al.* 1986; Webb and Gauld, 1987). Mean number of oestrogen-active follicles per ewe ($n=6$ per genotype) did not differ between heterozygous and non-carrier ewes (1.5 ± 0.8 and 0.7 ± 0.4 respectively).

Figure 9.1. The frequency (%) distribution of different size follicles from two genotypes (Fec^BFec^+ and Fec^+Fec^+) of the backcross population (Experiment 1).



Within each genotype, mean diameter of oestrogen-active follicles was not significantly different from mean diameter of the oestrogen-inactive follicle (Table 9.1.). There was no genotypic difference in mean (\pm s.e.m.) diameter of either oestrogen-active or oestrogen-inactive follicles (Table 9.1.) and so values were pooled across genotypes for both oestrogen-active and oestrogen-inactive follicles for further analyses. There was no strong linear relationship (ie. r was <0.5) between diameter and follicular fluid oestradiol concentration for either oestrogen-active or oestrogen-inactive follicles.

9.3.1.3. Follicular fluid inhibin

Geometric mean inhibin concentration in all follicles, irrespective of oestrogenic status, was significantly greater ($P<0.001$) in heterozygous ewes compared with non-carrier ewes (1566.8 ± 134.1 , 741.3 ± 66.9 ng/ml respectively).

Within each genotype, geometric mean inhibin concentration in oestrogen-active follicles was significantly greater ($P<0.01$) than in oestrogen-inactive follicles (Table 9.1.). There was no significant difference between heterozygous and non-carrier ewes in geometric mean (\pm s.e.m.) inhibin concentration in oestrogen-active follicles and so values were pooled for further analyses. There was no strong linear relationship (ie. r was <0.5) between mean inhibin concentrations and either diameter or oestradiol concentration in both oestrogen-active and oestrogen-inactive follicles. However, geometric mean (\pm s.e.m.) inhibin concentrations in oestrogen-inactive follicles were significantly higher ($P<0.001$) in heterozygous ewes compared to non-carrier ewes (Table 9.1.).

Table 9.1. Mean (\pm s.e.m.) diameter (mm), geometric mean (\pm s.e.m.) oestradiol concentration (ng/ml) and geometric mean (\pm s.e.m.) inhibin (ng/ml) in follicles dissected from heterozygous or non-carrier ewes from the backcross population (Experiment 1).

	Fec ^B Fec ⁺		Fec ⁺ Fec ⁺	
	Oestrogen- Active	Oestrogen- Inactive	Oestrogen- Active	Oestrogen- Inactive
Diameter	2.7 \pm 0.4 (n=9)	2.3 \pm 0.1 (n=81)	2.9 \pm 0.8 (n=4)	2.6 \pm 0.1 (n=100)
Oestrogen	95.5 \pm 22.8 ^e (n=9)	6.0 \pm 0.7 ^f (n=81)	74.1 \pm 14.0 ^g (n=4)	5.4 \pm 0.5 ^h (n=100)
Inhibin	3020 \pm 1042 ^a (n=9)	1445 \pm 105 ^b (n=81)	2884 \pm 1939 ^c (n=4)	708 \pm 66 ^d (n=100)

Across rows a versus b, c versus d $P < 0.01$, b versus d, e versus f, g versus h $P < 0.001$,

9.3.2. Experiment 2

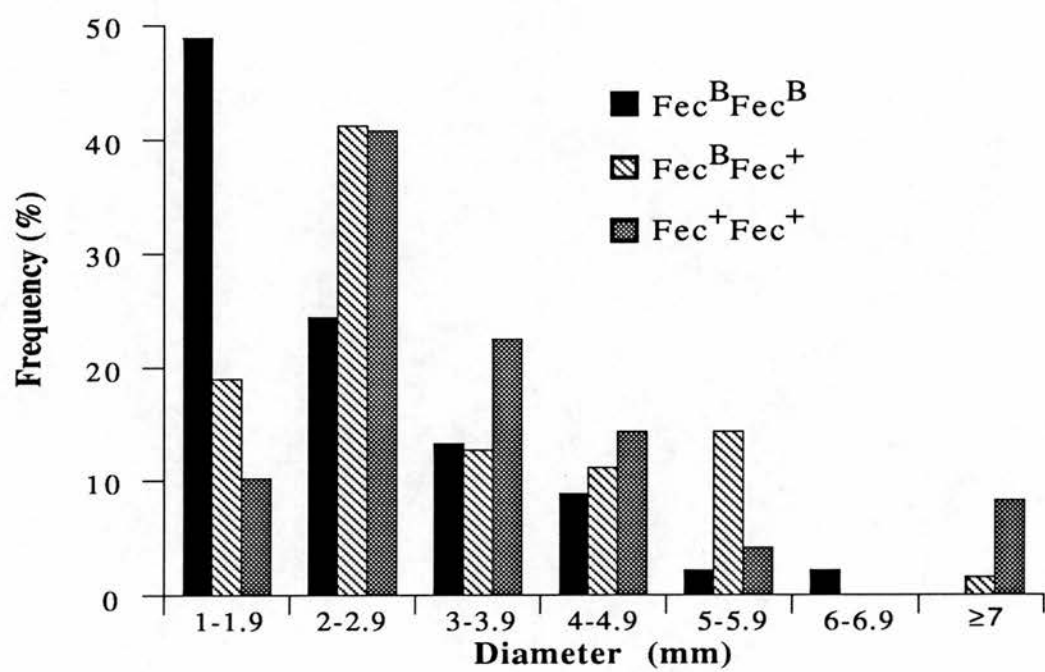
9.3.2.1. Follicle distribution

Mean (\pm s.e.m.) ovulation rate for homozygous ewes (ovulation rate 3.6 ± 0.7) was significantly greater in heterozygous ($P < 0.05$; ovulation rate 2.3 ± 0.2) and non-carrier ($P < 0.01$; ovulation rate 1.3 ± 0.2) ewes. Mean ovulation rate in heterozygous ewes was also significantly greater ($P < 0.01$) than in non-carrier ewes.

Mean (\pm s.e.m.) number of follicles per ewe (≥ 1 mm in diameter) was not significantly different between homozygous, heterozygous and non-carrier ewes (8.8 ± 1.1 , 11.9 ± 1.3 and 11.8 ± 1.2 respectively). The frequency distribution (%) of follicles on the basis of size is shown in **Figure 9.2.** and was significantly different ($P < 0.05$ and $P < 0.01$) between homozygous carriers compared to heterozygotes and non-carriers ewe (X^2 12.8. and 17.5 respectively). The frequency distribution of follicles on the basis of size was not significantly different between heterozygotes and non-carrier ewes.

Mean (\pm s.e.m.) number of oestrogen-active follicles per ewe was not significantly different between homozygous, heterozygous and non-carrier ewes (0.7 ± 0.3 , 0.4 ± 0.3 and 1.3 ± 0.4 respectively). For carriers of the Booroola fecundity gene, that is, homozygotes or heterozygotes, the mean number of oestrogen-active follicles per ewe were significantly ($P < 0.01$) lower than the respective genotypic mean ovulation rate. For non-carriers of the Booroola fecundity gene, mean ovulation rate was not significantly different from the number of oestrogen active follicles.

Figure 9.2. The frequency (%) distribution based of different size follicles from three genotypes ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$, $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$) of the F_2 population.



9.3.2.2. Follicular fluid oestradiol

For each genotype, mean diameter of oestrogen-active follicles was not significantly different from mean diameter of oestrogen-inactive follicles. There were no genotypic differences in the mean diameter of either oestrogen-active or oestrogen-inactive follicles (Table 9.2.).

Follicular fluid geometric mean oestradiol concentration in all follicles, irrespective of oestrogenic status, from either homozygous, heterozygous or non-carrier ewes, was not significantly different between genotypes (7.3 ± 1.1 , 8.2 ± 1.1 and 9.9 ± 1.6 ng/ml respectively). Mean (\pm s.e.m.) oestradiol concentration in both oestrogen-active and oestrogen-inactive follicles did not differ significantly between genotypes (Table 9.2.) and so genotypic values of either oestrogen-active or oestrogen-inactive follicles were pooled for further analyses. There was no significant correlation between follicular diameter and follicular fluid oestradiol concentration for either oestrogen-active or oestrogen-inactive follicles.

9.3.2.3. Follicular fluid inhibin

Geometric mean inhibin concentration in all follicles, irrespective of oestrogenic status, from homozygous, heterozygous and non-carrier ewes was not significantly different (957.2 ± 228.3 , 809.1 ± 152.4 and 885.1 ± 188.7 ng/ml respectively).

For each genotype, geometric mean inhibin concentration in oestrogen-active follicles was not statistically different from oestrogen-inactive follicles (Table 9.2.). Mean inhibin concentration in follicular fluid in both oestrogen-active or oestrogen-inactive follicles did not differ between genotypes and so values were pooled for either oestrogen-active

Table 9.2. The mean (\pm s.e.m.) diameter (mm), geometric mean (\pm s.e.m.) inhibin (ng/ml) and geometric mean (\pm s.e.m.) oestradiol concentration (ng/ml) in follicles dissected from ewes which were homozygous, heterozygous or non-carriers within the F₂ population (Experiment 2).

	Fec ^B Fec ^B		Fec ^B Fec ⁺		Fec ⁺ Fec ⁺	
	Oestrogen- Active	Oestrogen- Inactive	Oestrogen- Active	Oestrogen- Inactive	Oestrogen- Active	Oestrogen- Inactive
Diameter	2.2 ± 0.5 (n=4)	2.9 ± 0.3 (n=54)	2.1 ± 0.5 (n=4)	2.7 ± 0.2 (n=82)	2.5 ± 0.54 (n=8)	2.7 ± 0.2 (n=67)
Oestrogen	69.2 ± 13.0 ^a (n=4)	6.2 ± 0.9 ^b (n=54)	81.3 ± 15.3 ^a (n=4)	7.4 ± 0.9 ^b (n=82)	135 ± 22.1 ^a (n=8)	7.2 ± 1.6 ^b (n=67)
Inhibin	1349 ± 2074 (n=4)	933 ± 223 (n=54)	3162 ± 2006 (n=4)	759 ± 143 (n=81)	1123 ± 941 (n=8)	871 ± 208 (n=67)

Across rows a versus b P<0.001

or oestrogen-inactive follicles. There was no strong linear relationship ($r < 0.5$) between mean diameter and geometric mean inhibin concentration of oestrogen-active follicles or oestrogen-inactive follicles. For oestrogen-active follicles, there was a significant negative ($P < 0.05$) correlation ($r = -0.587$, $n = 16$) between geometric mean oestradiol concentration and geometric mean inhibin concentrations in follicular fluid, while a significant ($P < 0.001$) positive correlation ($r = 0.50$, $n = 201$) existed in oestrogen-inactive follicles.

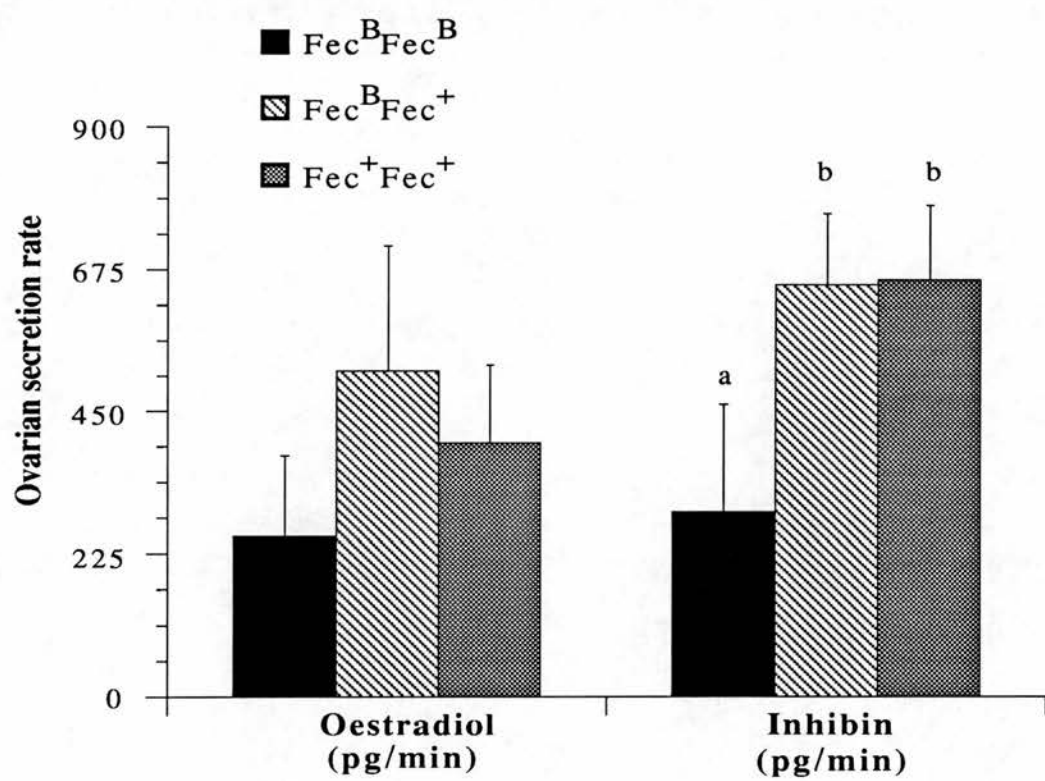
9.3.2.4. Ovarian secretion rate of oestradiol

Peripheral oestradiol concentrations were not measured in this study, but are relatively low during the luteal phase (< 4 pg/ml: Scaramuzzi and Land, 1978), when compared to the experimentally determined, ovarian venous oestradiol concentrations (Figure 9.3.). Thus ovarian secretion rate (pg/min) has been obtained from the product of ovarian venous oestradiol concentration and flow rate, without correction for peripheral oestradiol concentration. Mean oestradiol secretion rate did not differ between genotypes (Figure 9.3.).

9.3.2.5. Ovarian secretion of inhibin

Geometric mean inhibin concentrations in jugular venous samples did not differ between homozygous, heterozygous and non-carrier ewes (109.6 ± 20.9 , 101.9 ± 23.9 and 109.3 ± 12.6 pg/ml respectively) but were subtracted from the ovarian vein concentration prior to calculation of ovarian secretion rate. Mean ovarian secretion rate for inhibin (pg/min) was significantly lower in homozygous ewes compared to either heterozygous or non-carrier ewes ($P < 0.05$ respectively). However, mean ovarian secretion rate of inhibin was not significantly different between heterozygous and non-carrier ewes (Figure 9.3.).

Figure 9.3. Ovarian secretion rate of oestradiol (pg/min) and inhibin (pg/min), on day 7 of the oestrous cycle, in three genotypes ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$, $\text{Fec}^{\text{B}}\text{Fec}^{+}$ and $\text{Fec}^{+}\text{Fec}^{+}$) from the F_2 population (Experiment 2). In the figure, the columns indicated by a versus b, $P < 0.05$.



9.4. Discussion

Our previous studies have shown that peripheral FSH concentrations during the oestrous cycle are significantly higher in homozygous ewes compared to heterozygous or non-carrier ewes (Chapter 3). The finding that ovarian secretion rates of inhibin are significantly lower in homozygous ewes compared to the other two genotypes supports the hypothesis that inhibin and FSH are inversely related (McNeilly and Baird, 1989; Findlay *et al.*, 1990; McNeilly *et al.*, 1989). However, caution must be exercised since this finding only relates to one time point. Interestingly, McNatty *et al.* (1992), reported no genotypic differences in ovarian secretion rate of inhibin during the luteal phase, in pooled samples from Booroola Merino and Booroola Romney ewes, although comparisons of immunoreactive inhibin between genotypes have previously been compounded by the use of inappropriate controls, or by pooling samples from different strains (McNatty *et al.*, 1990).

The lack of genotypic differences in ovarian secretion rates observed in this study is similar to previous reports (Scaramuzzi *et al.*, 1983; McNatty *et al.*, 1985). Similarly, data in our study also found no genotypic differences in geometric mean oestradiol concentrations in follicular fluid obtained from all follicles in the F₂ population of Booroola Scottish Blackface ewes.

For both populations (backcross and F₂), the total number of follicles (>1mm in diameter) dissected per ewe did not differ between the genotypes. This agrees with other authors (Driancourt *et al.*, 1985) who report that the increased ovulation rate in Booroola ewes is not achieved through the availability of greater numbers of antral follicles. As follicles mature, they acquire increased numbers of LH receptors on the theca and granulosa

cells and the ability to secrete large amounts of oestradiol (England *et al.*, 1981; Webb *et al.*, 1981). During the follicular phase of the oestrous cycle, the number of oestrogen-active follicles is typical of the ovulation rate for that breed (see Webb *et al.*, 1985a for review; Driancourt *et al.*, 1988) and shows that LH responsive follicles are not limited to the follicular phase.

In this study, mean number of oestrogen-active follicles obtained from the F₂ population, during the luteal phase of the oestrous cycle, did not differ between the genotypes and was less than the observed ovulation rate for (homozygous or heterozygous) carriers of the Fec^B gene. For non-carriers from the F₂ population, there was no significant difference between the number of oestrogen-active follicles and ovulation rate. The reason for the reduced number of oestrogen-active follicles in homozygous and heterozygous carriers is unclear, although it is possible that during the early to mid-luteal phase of the oestrous cycle, there are less oestrogenic follicles present due to lower peripheral concentrations of LH. However, administration of a single 750 i.u. i.m. hCG in the luteal phase results in a 68% increase in ovulation rate in all Booroola genotypes, providing evidence that mature follicles are present during this stage of the oestrous cycle (Chapter 3). Alternatively, this low number of oestrogen-active follicles may reflect a deficiency in the classification of follicles, particularly for breeds/strains which exhibit high ovulation rates. This study found little evidence of a bimodal distribution of two populations of follicles, based on oestradiol production as previously found during the follicular phase of the cycle (Webb *et al.*, 1989). Further evidence that follicles from the luteal phase of the oestrous cycle may require a different classification system comes from *in vivo* evidence which suggests a greater steroidogenic response to follicles to LH during the luteal phase (Baird, 1978; Baird *et al.*, 1981; Campbell *et al.*, 1990b). Also,

oestrogenic follicles collected during the follicular phase secrete more oestradiol and testosterone *in vitro* than their luteal phase counterparts (Picton, 1989). However *in vitro* evidence suggests that oestrogen-active follicles, on a per cell basis, do not have higher aromatase activity or produce more androgen during the follicular phase (McNatty *et al.*, 1986).

In the backcross population, mean number of oestrogen-active follicles obtained during seasonal anoestrus did not differ between genotypes. Induction of ovulation during seasonal anoestrus by administration of a single i.m. injection of hCG within the backcross population (see section 3.3.) is comparable to the number of oestrogen-active follicles found in this study. These results are similar to previously published data in different breeds, and confirm that the mechanism controlling the number of mature follicles is functional during seasonal anoestrus (Webb *et al.*, 1985b; Webb *et al.*, 1992).

For each genotype within both the backcross and F₂ population, the mean diameter of oestrogen-active follicles did not differ from mean diameter of oestrogen-inactive follicles. This is in agreement with other prolific breeds of sheep, such as the Finnish Landrace, where mean diameter of oestrogen-active follicles obtained during seasonal anoestrus were not significantly different from oestrogen-inactive follicles (Webb *et al.*, 1992b), probably since the ovary contains a mixed population of both small and large oestrogenic and non-oestrogenic follicles (Webb *et al.*, 1982; McNatty *et al.*, 1982). In addition, there was no strong linear relationship between antral fluid concentration of oestradiol and size of either oestrogen-active or oestrogen-inactive follicles. This lack of correlation between antral fluid oestradiol concentration and follicle size has also been observed in the less prolific Suffolk ewes (England *et al.*, 1981).

McNatty *et al.* (1992) found no relationship between follicular fluid inhibin concentrations and follicular diameter, although follicular fluid inhibin concentrations have been reported to be higher in larger follicles (>3.5mm in diameter) compared to smaller follicles (Tsonis, Quigg, Lee, Leversha, Trouson and Findlay, 1981). Such discrepancies may be the result of antisera cross-reacting with free α -subunit (see Knight, 1991 for review). In our study, using IRMA, which has minimal cross-reactivity with free α -subunit, there was no strong linear relationship between antral fluid concentration of inhibin and size of either oestrogen-active or oestrogen-inactive follicles. The extent to which subunits are present in follicular fluid or in the peripheral circulation is at present unknown (McNatty *et al.*, 1992), although the adrenal cortex possesses mRNA for free α -subunit and appear to produce free unprocessed high molecular weight α -inhibin subunit (Crawford, Hammond, Evans, Coghlan, Haralambidis, Hudson, Penschow, Richards and Tregar, 1987; Webb, Baxter, Brooks and McNeilly, 1992).

Within both genotypes from the backcross population, geometric mean inhibin concentration was significantly higher ($P < 0.01$) in oestrogen-active follicles compared to oestrogen-inactive follicles. However, this was not the case in the F₂ population. The difference between the two populations in the relationship between follicular fluid oestradiol and follicular fluid inhibin concentrations is difficult to interpret, unless there was a difference between seasonal anoestrus and the breeding season.

Unlike oestradiol, genotypic differences were found in ovarian secretion rates of inhibin, although this was not reflected in antral fluid inhibin concentrations. The reasons for this apparent discrepancy are not

clear, since there were no genotypic differences in peripheral inhibin concentrations. However, it may be remembered that follicular fluid provides a measure of follicular inhibin production, whereas ovarian secretion rate is a measure of ovarian inhibin production over a shorter time period. Clearly, more study is required to investigate possible pulsatile release of dimeric inhibin and the mechanisms which regulate its production and secretion during seasonal anoestrus and the breeding season. The lower secretion rate of immunoreactive inhibin in homozygous carriers compared to either heterozygotes or non-carriers does however agree, with higher peripheral FSH concentrations in homozygous carriers compared to the other two genotypes (Chapter 3). At present, the proportion of peripheral inhibin which is free α -subunit or biologically active dimeric form is not known.

In conclusion, ovarian secretion rates of inhibin were significantly lower in homozygous ewes compared to either heterozygous or non-carrier ewes. In view of these genotypic differences in ovarian secretion rates of inhibin, it was surprising to find no genotypic differences in follicular fluid inhibin concentrations. Despite no genotypic differences in ovarian secretion rates, or follicular fluid concentrations, of oestradiol, the lower ovarian secretion rates of inhibin in homozygous carriers, compared to the other two genotypes, may account for increased peripheral FSH concentrations.

CHAPTER 10

10.1. General Discussion

The work in this thesis has investigated gene specific differences in FSH, inhibin, oestradiol and IGF-1 as possible agents in promoting prolificacy in Booroola Scottish Blackface ewes.

Investigation of FSH being responsible for controlling genotypic differences in ovulation rates in ewes possessing the Booroola gene have been reported previously, but the situation has been complicated by the use of animals which were of an unknown genetic background and in some cases comparing animals of different strains. The establishment of breeding lines has enabled animals to be chosen whose genetic background is well defined and whose genetic relationship to members within the same experimental group can be carefully regulated. An additional advantage of using animals which have been carefully chosen from discrete breeding lines is that it provides a rare opportunity to study the physiology of two populations that differ only in the Booroola gene and its related genes.

The effect of FSH in differentially controlling follicular development, culminating in genotypic differences in mean ovulation rates, was investigated in three ways; firstly, by measurement of peripheral FSH concentrations (Chapters 3 and 4); secondly, by use of an *in vitro* culture system (Chapters 5 and 6) and finally by use of an *in vivo* model using ovarian xenografts placed into ovariectomized SCID mice (Chapter 7).

Examination of peripheral FSH concentrations in two populations (F₂ or backcross population) of Booroola Scottish Blackface ewes, during the oestrous cycle and the non-breeding season, indicated that FSH was not wholly responsible for controlling genotypic differences in ovulation rate (Chapter 3). Interestingly, in both populations, approximately 80% of ewes were found to ovulate in response to a single i.m. injection of hCG, confirming that the mechanisms controlling ovulation are still functional in the non-breeding season (Webb *et al.*, 1985; Driancourt and Fry, 1990). These data showed that, at least for homozygous carriers of the gene, with higher ovulation rates, genotypic mean FSH concentrations were higher compared to non-carrier ewes, in agreement with McNatty *et al.* (1987; 1989). It is important to note, however, that differences in the immunassayable concentrations of the hormone may not reflect differences in biological activity or clearance rate, which may occur as a result of differences in the secretion/synthesis of different glycosylated forms of FSH (Montgomery *et al.*, 1992). Further investigation into expression, synthesis/secretion and regulation of these subunits of FSH may prove a useful avenue for future work. Despite these differences, mean peripheral FSH concentrations could not fully account for the genotypic differences in ovulation rates, since no significant differences in FSH were found between heterozygous and non-carrier ewes from either population, despite significant differences in ovulation rate.

After ovariectomy, FSH concentrations were elevated compared to intact animals, for all genotypes in both populations of ewes (Chapter 4). Interestingly, differences in FSH concentration, relative to each genotype within that population, were still maintained after ovariectomy. Although the ovary may regulate the release of FSH through negative feedback mechanisms, it appears to do so to the same degree for each genotype,

such that relative differences in mean FSH concentration between genotypes that are seen in intact animals are still maintained post-ovariectomy. These data show that genotypic differences in peripheral FSH concentrations occur independently of ovarian hormones in agreement with McNatty *et al.* (1990). The genotypic differences in peripheral FSH following ovariectomy are probably due to differences in pituitary gland release, although this has not been confirmed. However, peripheral FSH concentrations did not differ between heterozygous and non-carrier ewes in intact or ovariectomized animals, suggesting that the Booroola fecundity gene may also exert its effect at the level of the ovary.

Since our studies have shown that FSH concentrations are similar between heterozygotes and non-carrier ewes, but that genotypic differences in ovulation rate are still maintained, it seems reasonable to suggest that there may be a difference in follicular responsiveness (in terms of growth or oestradiol production) to FSH. An *in vitro* culture system for small antral follicles was validated (Chaper 5) to enable this hypothesis to be tested. However, the results showed no genotypic differences in oestradiol production or growth in response to FSH (Chapter 6). The possibility that genotypic differences in follicular responsiveness to FSH, and/or other factors, occurs at the latter stages of development cannot be excluded, particularly since ewes possessing the Booroola fecundity gene are thought to have an extended recruitment phase and lower intensity of selection (Driancourt *et al.*, 1985). It is thought that the Booroola fecundity gene also causes follicles to mature at an earlier size compared to non-carriers of the gene (Scaramuzzi and Radford, 1983) and our data for small antral follicles, shows that the Booroola fecundity gene may exert its effect upon the size of the follicle at this early stage of differentiation.

The major disadvantage of the *in vitro* culture system established for ovine follicles (Chapters 5 and 6) was that growth and oestradiol production did not continue through to the final stages of development. A novel *in vivo* follicle culture system, using ovariectomized SCID mice transplanted with ovine ovarian grafts containing primordial and small follicles (Chapter 7), was investigated to determine if follicular growth could continue to more advanced stages of development and if so, whether genotypic differences existed. The xenografting technique produced follicles which secreted oestradiol and developed to the antral stage (where all follicles were <2mm in diameter), although not to the Graafian stage. The production of small antral follicles within the xenograft tissue was variable and so genotypic differences in follicular development could not be ascertained. The ovariectomized SCID mouse model may however, be a useful tool to investigate factors controlling the initiation of growth of primordial follicles.

Since peripheral FSH measurements did not totally explain genotypic differences in ovulation rate and FSH *in vitro* did not cause genotypic differences in either growth or oestradiol production by small antral follicles, this led to speculation about the involvement of other factor(s) in the control of genotypic differences in folliculogenesis. A prime candidate is IGF-1, an amplifier of FSH action *in vitro* (Adashi *et al.*, 1986b; Webb and McBride, 1991) and another is inhibin, an inhibitor of FSH release *in vivo* (Martin *et al.*, 1988; Webb, 1988).

Peripheral IGF-1 concentrations within the F₂ population significantly increased during the follicular phase of the oestrous cycle in the ewe, suggesting acute regulation of IGF-1 by the ovary, which is itself not a major producer of IGF-1. It may be possible that oestradiol is

responsible for the increase in IGF-1 observed during the follicular phase, although whether the ovary contributes to this increase in peripheral IGF-1 is doubtful since the liver is the major source of IGF-1 production. Also, peripheral IGF-1 concentrations did not differ between intact and ovariectomized ewes, confirming the ovary is not a major source of peripheral IGF-1.

There were no genotypic differences in peripheral IGF-1 concentrations; during seasonal anoestrus; from day -2 until day 7 of the oestrous cycle, or following ovariectomy, in the F₂ population. Further evidence that peripheral concentrations of IGF-1 are not associated with ovulation rate has recently been shown in Finnish Landrace ewe lambs (Spicer *et al.*, 1993). It is at present unknown whether genotypic differences in ovarian IGF-1 production exist in response to the genotypic differences in peripheral FSH (Chapters 3 and 4), but that this effect is masked by the greater production of hepatic IGF-1. Further studies should investigate local production of IGF-1 and IGFBPs between the genotypes and the possible synergistic action of IGF-1 with FSH, as regulatory mechanisms which may culminate in genotypic differences in ovulation rate.

Investigation of ovarian secretion rates of inhibin, within the F₂ population, revealed homozygous ewes to have significantly lower concentrations than either heterozygotes or non-carrier ewes. This finding supported the hypothesis that peripheral FSH and inhibin concentrations were inversely related to each other (Martin *et al.*, 1986; Martin *et al.*, 1988; Campbell *et al.*, 1990a; Findlay *et al.*, 1990; Baird *et al.*, 1991). Interestingly, ovarian secretion of oestradiol, shown to act synergistically with inhibin to inhibit FSH release (Martin *et al.*, 1988; Webb, 1988) did not differ between genotypes. In view of genotypic differences in peripheral inhibin concentrations, it was therefore

surprising to find no genotypic differences in follicular fluid concentrations of inhibin. The reason for this apparent discrepancy may be due to the different assay methods employed. Assay of peripheral measurements of inhibin are based on antibodies raised to the α -subunit, thereby measuring all forms of inhibin, whilst assay of follicular fluid samples are based on antibodies raised to both the α - and β -subunits, which therefore measure dimeric inhibin. It is interesting that the synthesis of α -inhibin subunit does not differ between genotypes, although genotypic differences ($Fec^B Fec^B > Fec^+ Fec^+$) in the synthesis of the β_A subunit have been found during the luteal phase of the oestrous cycle (Fleming, Tisdall, Greenwood, Hudson, Heath and McNatty, 1992). Further work is required to clarify whether this difference in the synthesis of the β_A subunit is a secondary effect of Fec^B expression or whether the Fec^B gene is genetically linked to the β_A subunit (Fleming *et al.*, 1992). It is at present unknown whether mRNA expression of these subunits varies throughout the oestrous cycle.

The work in this thesis has attempted to clarify the roles of FSH, inhibin and IGF-1 in controlling follicular growth and ovulation rate in ewes possessing the Booroola fecundity gene by use of defined breeding lines, enabling animals to be chosen which were of a known genetic background. In conclusion, there are genotypic differences in peripheral FSH concentrations during the breeding and non-breeding seasons. However, FSH does not appear to be wholly responsible for controlling the genotypic differences in ovulation rate and genotypic differences in ovarian secretion of inhibin, although not oestradiol, have been implicated. It is unknown whether the lower ovarian secretion rate of inhibin in homozygous ewes, compared to heterozygous or non-carriers ewes, is the cause or effect of genotypic differences in ovulation rate. The

Booroola fecundity gene, responsible for controlling genotypic differences in ovulation rate, appears to be exerting its effect at two sites, that is, at the level of the pituitary gland and at the level of the ovary. The lack of genotypic differences in the growth and steroidogenesis of small antral follicles in response to FSH *in vitro* (Chapter 6) does not exclude the possibility that genotypic differences in either follicular responsiveness or sensitivity to FSH may exist towards the latter stages of development. More emphasis should be placed on investigations into factors which may be important in controlling the extended recruitment phase, lower incidence of selection and the ability of follicles to 'wait' for ovulation (Driancourt *et al.*, 1985). In addition, there have been no published reports of the physiology of the brain-thyroid or brain-adrenal axis in ewes possessing the Booroola gene. Further investigations are required to determine which, if any, of the sites of action of the Booroola fecundity gene is the predominant one and what the exact mechanisms are which differentially regulate follicular growth and steroidogenesis between the genotypes.

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